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1995

Acta Microbiologica et Immunologica Hungarica

VOLUME 42, NUMBER 1, 1995

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ACTA MICROBIOL. ET IMMUNOL. HUNG. 42 (1) 1-140 (1995) HU ISSN 1217-8950

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A QUARTERLY OF THE HUNGARIAN
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Acta Microbiologica et Immunologica Hungarica publishes reviews and original papers on microbiology and immunology in English

Acta Microbiologica et Immunologica Hungarica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1117 Budapest, Prielle K. u. 19-35

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Subscription price for Volume 42 (1995) in 4 issues US\$ 98, including normal postage, airmail delivery US\$ 20.00.

Acta Microbiologica et Immunologica Hungarica is abstracted/indexed in Abstracts of World Medicine, Biological Abstracts, Chemical Abstracts, Chemie-Information, Current Contents-Life Sciences, Excerpta Medica database (EMBASE), Index Medicus

"This periodical is included in the document delivery program THE GENUINE ARTICLE of the Institute of Scientific Information, Philadelphia. The articles published in the periodical are available through *The Genuine Article* at the Institute for Scientific Information, 3501 Market Street, Philadelphia PA 19104."

CONTENTS

B Cell Specific Natural IgM Autoantibodies: Multifunctional Regulators of the Humoral Immune Response (A Review)	
Uher, F., Kiss, K., Rácz, J., Mihalik, R., Puskás, É., Alonso, M. E.	3
Growth of Untreated and Radiation-Damaged <i>Listeria</i> as Affected by Environmental Factors	
Farkas, J., Andrásy, É., Mészáros, L., Bánáti, D.	19
Production and Some Characteristics of β -Glucosidase in <i>Diaporthe (Phomopsis) Helianthi</i>	
Peričin, D., Jarak, M.	29
Epifluorescent Microscopy of Earthworms' Intestinal Bacteria	
Krisztófek, V., Pižl, V., Ravasz, K.	39
Bacteria Adherent to the Hindgut of Terrestrial Isopods	
Drobne, D.	45
Hybrid Process for Ethanol Production from Rice Straw	
Chadha, B. S., Kanwar, S. S., Saini, H. S., Garcha, H. S.	53
Effect of Some Environmental Factors on <i>Rhizobium</i> and <i>Bradyrhizobium</i> Strains	
Bayoumi, H. E. A. F., Bíró, B., Balázs, S., Kecskés, M.	61
Simultaneous Saccharification and Fermentation of Rice Straw into Ethanol	
Chadha, B. S., Kanwar, S. S., Garcha, H. S.	71
A Simple and Rapid Semiquantitative Method for Measuring Cellulase Activity in Agar Media	
Jakucs, E., Várallyay, É.	77
HLA Associated Cytotoxic Antibody Production in Dialyzed Chronic Uraemic Patients	
Kovanecz, I., Petri, I., Kaiser, G.	81
Diversity in the Antigenic Structure of Different Hexon Types among the Members of Adenovirus Subgenus D	
Ádám, É., Nász, I., Lengyel, A.	85
Annual Meeting of the Hungarian Society for Microbiology	93
Erratum	141

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B CELL SPECIFIC NATURAL IgM AUTOANTIBODIES: MULTIFUNCTIONAL REGULATORS OF THE HUMORAL IMMUNE RESPONSE

(A REVIEW)

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(Received March 28, 1994)

(Accepted March 31, 1994)

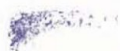
Introduction	3
Anti-lymphocyte reactivity of natural autoantibodies	4
The phenomenon	6
Properties and characteristics of the regulatory IgM	6
The mechanism of the regulatory IgM mediated inhibition of polyclonal B cell growth	7
The B cell specific autoantibody does not inhibit memory B cell responses	8
Appearance and expansion of the regulatory IgM producing B cell clones during ontogeny	9
The regulatory B cells belong into the B1 developmental lineage	9
Implications for immunopathology: autoimmune diseases and immunodeficiencies	12
Concluding remarks and speculations	14
References	16

Introduction

Over the past two decades, it has been clearly demonstrated that a high number of autoreactive B cells are present in normal humans and mice. Two to fifty per cent of all the monoclonal immunoglobulin-secreting clones, prepared from different lymphoid organs [1–8], as well as polyclonally activated normal B lymphocytes [9, 10] secrete natural autoantibodies. In humans, B cells synthesizing autoreactive IgM and IgA antibodies predominate but IgG are also found, whereas in mice, IgM-producing B cells are almost exclusively seen [11]. Most natural autoantibodies, directly encoded by the germ line and subjected to practically no mutations [12–14], are capable of

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recognizing several self and non-self antigens including molecules conserved throughout the course of evolution, for example, actin, myosin, tubulin, DNA, and phosphatidylserine, i.e., they are polyreactive [6, 7, 10, 15]. Moreover, normal human serum and those from other animal species, also contain a huge amount of polyreactive natural autoantibodies [11, 16]. Monoreactive natural autoantibodies, however, have been found too [6, 17, 18].

These and additional experimental observations bring us to the concept according to which one of the fundamental laws of the immune system is the active recognition of self constituents. Among themselves, natural autoantibodies form a dense idiotypic network [19–21], the so-called “central immune system” [22] or “immunological homunculus” [23, 24], whose important role in the regulation of the immune responses has been clearly demonstrated. In addition, they are able to interact with several other circulating, membrane exposed, and intercellular antigens [2, 6, 14–16, 25]. Therefore, it is highly probable that the natural autoantibodies and the B cells that synthesize them participate in much more specific reactions involved in immune responses.

Anti-lymphocyte reactivity of natural autoantibodies

In fact it is well established that many natural autoantibodies are able to react with neoplastic [26], as well as normal cells, including thymocytes, T and B lymphocytes [27–29]. Moreover, they often inhibit spontaneous and/or mitogen induced cell proliferation both in vivo [30] and in vitro [31]. Recently we could confirm and extend these observations [32, 33, and unpublished data]. As shown in Table I, supernatant culture fluid from normal murine splenic B lymphocytes stimulated with LPS for 96 h (B-SN) and the affinity purified IgM fraction of B-SN are able to inhibit the mitogen induced proliferation of freshly isolated normal mouse, rat, and human B cells in vitro. In contrast, ConA or PHA induced normal T cell growth was never affected by the B-SN. Furthermore, the expansion of all neoplastic B cell lines and clones tested, including pre-B (NSF-5, PD-31), immature B (WEHI-231), mature B (2PK-3, Bjab, B41, Raji, Ramos), and plasma cells (Sp2/0, IR202) was suppressed in this experimental system. The growth of some (BWS147G1.4, Jurkat), but not all (EL-4.IL-2, H-9, MOLT-4) of the neoplastic T cell lines was also inhibited by the natural IgM autoantibody containing supernatant. Monocyte (U937), macrophage (P388D1.IL-1), melanoma (B16, HT-168.M1), and lung carcinoma (A549) cell proliferation, however, was unaffected by the natural autoantibodies.

These and additional observations [31–33] suggested that the culture supernatant of LPS stimulated murine splenic B lymphocytes might contain a B cell specific regulatory IgM autoantibody.

Table I

Natural IgM autoantibodies proliferation in vitro are able to inhibit lymphocyte

Species	Cell type	Name	Inhibition of cell proliferation ^a	
			B-SN ^b	IgM ^c
Mouse	T	ConA stimulated normal	—	—
		EL-4.IL-2	—	—
		BWS147G1.4	++	++
	B	LPS stimulated normal	++	++
		NSF-5	++	++
		PD-31	+++	+++
		WEHI-231	++	++
		2PK-3	++	++
		Sp2/0	++	++
	Granulocyte	WEHI-3	++	NT ^d
	Macrophage	P388D1.IL-1	+	—
	Melanoma	B16	—	—
Rat	T	ConA stimulated normal	—	—
	B	PWM stimulated normal	++	++
		IR202	+++	+++
		Clones isolated from IR202	+++	+++
Human	T	PHA stimulated normal	—	—
		H-9	—	—
		MOLT-4	—	—
		Jurkat	++	++
	B	PWM stimulated normal	++	++
		Bjab	++	++
		B41	++	++
		Raji	++	++
		Ramos	++	++
	Monocyte	U937	—	—
	Melanoma	HT-168.M1	—	—
	Lung carcinoma	A549	+/-	—

^a +++ >80%, ++ 50–80%, + 30–50% inhibition of ³H-thymidine incorporation.^b Supernatant culture fluid (25% v/v) from murine splenic B cells incubated with LPS for 96 h^c Affinity purified IgM fraction (50 µg/ml) of B-SN^d Not tested

The phenomenon

The idea that a portion of B cells has the capacity to suppress both the polyclonal and antigen-specific responses of other B lymphocytes when transferred to cultures of fresh splenic B cells is an old one [34]. So-called "suppressor B cells" occur spontaneously and their activity may increase with age. Their activity can be markedly enhanced by B cell mitogens, including LPS [35, 36], or by lymphokines [37]. It was also shown that suppressor B cells mediate their effects via a soluble substance which they release into the culture medium. The inhibitory mediator, however, has never been precisely characterized [37].

One of our main observations, therefore, is that this soluble substance is simply a B cell-specific natural IgM autoantibody. First, the B cell growth-inhibitory material in the culture supernatants of murine splenic B lymphocytes stimulated with LPS for 96 h is heat-resistant (60 min, 56 °C), sensitive to trypsin treatment, and has a high molecular weight (near 1000 kDa), i.e. it is a large protein or glycoprotein molecule. Second, the inhibitor specifically adsorbed on, and eluted from, Sepharose 4B beads coated with anti- μ or anti-Ig antibodies. This finding directly implicates a natural IgM antibody in the suppression [33].

When B-SN or the affinity purified IgM fraction of the supernatant is added to fresh B cells, the inhibition is: (i) dose-dependent, (ii) persists throughout the entire culture period (4 days in general), (iii) independent from the number and proximity of B cells in the test culture, (iv) affect both the LPS, Fc-fragment of human IgG1, dextran-sulfate, anti- μ antibody, anti- μ antibody plus lymphokines, and PMA plus Calcium ionophore induced proliferation of B lymphocytes, and (v) neither species, nor MHC restricted [32, 33, and Table I].

Properties and characteristics of the regulatory IgM

An important question which always arises when dealing with IgM autoantibodies derived from polyclonally activated B cells is whether these antibodies are multi- or polyspecific immunoglobulins directed against highly conserved self epitopes, often binding ligands of varying chemical composition with low affinity [38–40]. Our results demonstrate, however, that these "sticky" IgM antibodies are not responsible for the autocrine B cell growth-inhibition. The regulatory IgM cannot bind to murine IgG or IgM (native or aggregated), BrMRBC, SRBC, thymocytes, DNA, SpA, or OVA; i.e., it seems to be not polyreactive. On the other hand, IgM preparations from pooled normal serum samples and monoclonal IgM antibodies with known specificity are unable to inhibit polyclonal B lymphocyte proliferation, i.e., the B cell growth-inhibitory activity is not a property of any collection of IgM molecules [41].

Attempts to identify the molecule recognized by the regulatory IgM, however, have so far been unsuccessful. It appears to be a B cell surface structure distinct from sIg, MHC class I and class II, Ly-1 (CD5), B220 (CD45R), Lyb-2.1 (CD72) antigens, transferrin and Fc γ receptors [33, 41].

Moreover, it does not seem to be a differentiation antigen of B cells since we found that it is expressed on all pre-B, mature B, and the plasma cell lines tested (Table I). Potential candidates as the target of this B cell specific regulatory IgM include the receptor for an autocrine growth-factor produced by activated B lymphocytes and B cell blasts [42], or a component of the murine analogue of the human CD19-CD21(CR2)-TAPA-1-Leu-13 complex [43]. The anti-proliferative effect of monoclonal anti-CD19 [44, 45], anti-TAPA-1 [46], and anti-Leu-13 [47] antibodies have been shown in several laboratories. Moreover, both anti-TAPA-1 and anti-Leu-13 antibodies caused aggregation in the tested B cells [46, 47]. Similar properties had been found for our regulatory IgM. Of course, the autoantibody may inhibit B cell growth through as yet undefined member of the tetraspan membrane protein family.

In addition, the fact that reduced and gel-filtered (monomeric) IgM was not biologically active suggests that an extensive cross-linking of this surface structure is required for the inhibition.

The mechanism of the regulatory IgM mediated inhibition of polyclonal B cell growth

We also investigated the mechanism of this autoantibody-mediated suppression of B lymphocyte proliferation in terms of cell cycle progression. We found that the regulatory IgM did not affect the entry into G₀* of LPS stimulated B lymphocytes as assessed by the increased expression of I-A antigens. Events that characterize the early G₁ phase (G_{1A}), such as cell enlargement and increased RNA synthesis, occur whether the autoantibody is present or not. In contrast, events that mark the G_{1B} phase, such as further cell enlargement and late RNA synthesis are inhibited by the regulatory IgM. Moreover, a significant part of the cells fail to incorporate ³H-thymidine and fail to progress through S, G₂, or M in the presence of the regulatory IgM, as revealed by their DNA content. Therefore, our work points toward a precise stage of the early G₁ phase at which the autoantibody inhibits further progression of mitogen stimulated B cells [48]. This possibility was also confirmed by another observation that B-SN does not affect ³H-thymidine uptake when added 24 h after LPS, i.e., when cells have passed the G_{1A} phase or are already synthesizing DNA [32].

These data suggest that the stage of the G₁ phase at which the regulatory IgM inhibit further progression of activated B lymphocytes through their cycle may be identical with a "restriction point" determined by others [49-51] and/or with the "commitment period" proposed by DeFranco et al. [52] in the cycle of mitogen stimulated murine B cells. This would afford an early control point in the replicative cycle to guard against unlimited cell proliferation in response to polyclonal activators.

Another important issue is that a prolonged stay at this point in the cycle would increase the capability of these cells to perform a specialized function, e.g., antigen presentation. As anticipated, we found that the increased antigen presenting capacity of LPS-stimulated B lymphocytes, first described by Krieger et al. [53], was not affected by exposure to the regulatory IgM [41]. Therefore, we propose that this autoantibody may direct mitogen-stimulated B cells to stop proliferating; and may play a

simultaneous accessory role in permitting up-regulation of I-A expression, thus enhancing the ability of these cells to present antigen and receive T cell help during heavy bacterial infections in vivo [54].

The B cell specific autoantibody does not inhibit memory B cell responses

On the basis of the above results emerged the question whether the regulatory IgM is able to affect the differentiation of B lymphocytes during polyclonal and/or antigen-specific immune responses? We found that this IgM inhibits not only the proliferation of murine splenic B cells, but also their IgM secretion during LPS-induced polyclonal, as well as antigen (FITC-KLH)-specific primary antibody responses. In contrast, secondary antibody (mainly IgG1) production of hapten (FITC)-specific B cells were affected by the regulatory IgM neither during their restimulation with LPS, nor in the presence of carrier (KLH)-specific T lymphocytes in vitro [55, and Fig. 1]. Therefore, whereas newly emerging naive B cells are highly susceptible, memory B cells appear completely resistant to inactivation by the autoantibody.

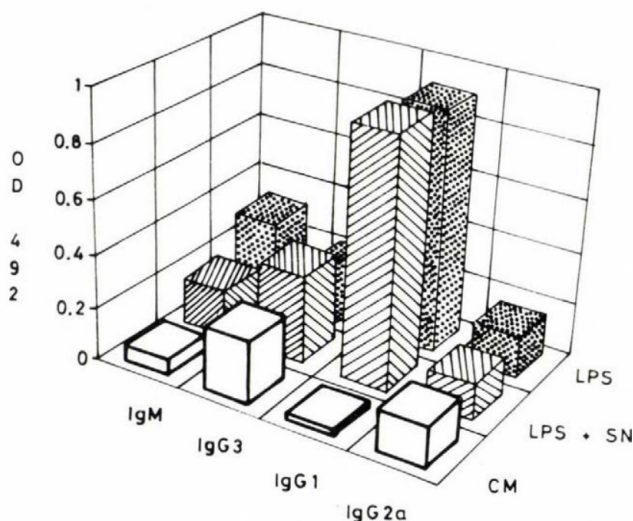


Fig. 1. FITC-specific secondary antibody response in the presence or absence of the regulatory IgM. DBA/2 mice were immunized with 50 µg of FITC-KLH and a secondary challenge was added 8 weeks later. Splenic B cells were then isolated and cultured at 2×10^6 cells/wells in 1 ml of complete medium either alone, or with LPS (10 µg/ml), or with LPS plus B-SN (12% v/v) for three days in vitro. The level of specific IgM, IgG3, IgG1, and IgG2a antibodies were determined by isotype-specific indirect ELISA using FITC-BSA coated plates. Data are representative of three similar experiments

This difference may indicate distinct signaling characteristics among B cells involved in primary vs those in secondary antibody responses. Alternatively, this might reflect one type of heterogeneity within splenic B cell populations. For example, it has been suggested that HSA (heat-stable antigen) expression distinguishes lineages corresponding to primary vs memory B cell progenitors, since 30 to 40% of MHC II and B220 (CD45R) positive cells with highest levels of HSA appear incapable of generating memory clones [56–58]. Regardless of basis, the lack of autoantibody-mediated inhibition during secondary anti-hapten response shows an important new feature of memory B cells.

Appearance and expansion of the regulatory IgM producing B cell clones during ontogeny

Since we have described a subpopulation of regulatory B cells, it seemed appropriate to investigate its establishment in ontogeny, particularly in view of the B1 lineage B cell predominance in the neonate [59–61]. Thus, splenic B lymphocytes from newborn (6 days old), young adult (2–3 months old), and old (more than 1 year of age) Balb/c mice were placed in culture and stimulated with LPS. At 24 h intervals, the supernatants were harvested and assayed for their growth-inhibitory activity on a second culture of mitogen stimulated B lymphocytes (Fig. 2A), and for their total IgM content by indirect ELISA (Fig. 2B). In parallel, the frequency of the regulatory B cell precursors among LPS-reactive splenic B cells were determined by limiting dilution analysis (LDA) (Fig. 2C). As can be seen in Fig. 2C, the frequency of regulatory B cell precursors is almost constant from as early as day 6 up to three month of age (1:11300 vs 1:8900). In old animals, however, the frequency of these B cell precursors in the spleen is 2–3 times increased, as compared with young adult mice (1:3800 vs 1:8900). It would appear, therefore, that the regulatory IgM-producing B cells are already present in the newborn, and they are positively selected in normal individuals during aging. Similar ontogenic development of other self-reactive B cell clones, particularly those constituting the neonatal idiotype network, have been described earlier [20, 29, 62].

Interestingly, despite the almost adult frequency of regulatory B cell precursor in newborns, culture supernatants of LPS-stimulated splenic B lymphocytes from newborn mice did not suppress B cell growth (Fig. 2A). However, this fact could be easily explained by the very low amount of IgM secreted by these B cells in response to LPS (Fig. 2B).

The regulatory B cells belong into the B1 developmental lineage

Taking as criteria for B lymphocyte activation the density of cells and their ability to spontaneously produce the regulatory IgM, we show that regulatory B lymphocytes in normal young adult mice are not only present in the spleen and

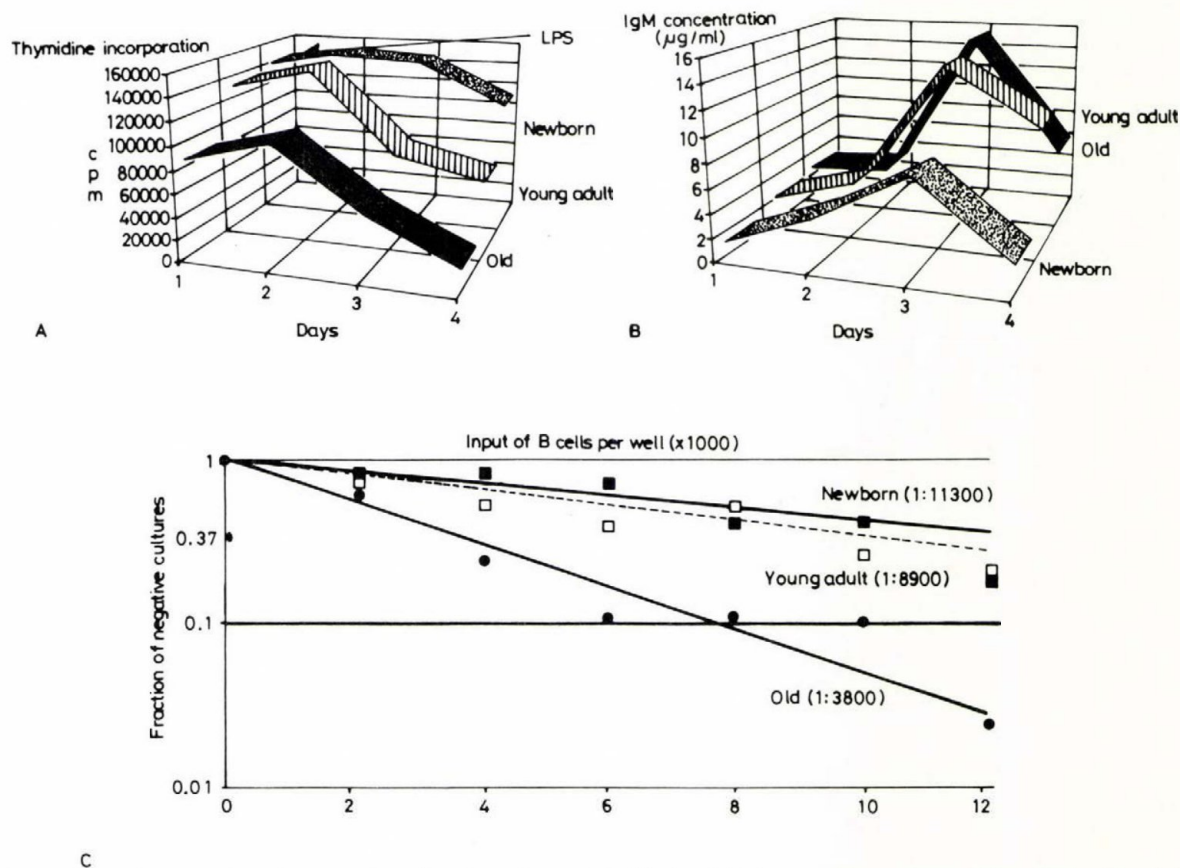


Fig. 2. Ontogenetic development of regulatory B cells in the spleen of normal Balb/c mice. (A) Kinetics of the regulatory IgM production by splenic B cells from newborn (6 day old), young adult (2–3-month-old), and old (>1 year old) animals. The B cells were cultured at 2×10^6 cells/ml with LPS (20 $\mu\text{g/ml}$) for 96 h. Aliquots of supernatants were collected each day and tested for their inhibitory activity. For this, freshly isolated B cells were cultured (2×10^5 /well) in 0.2 ml CM with LPS (10 $\mu\text{g/ml}$) plus 25% v/v of each type of supernatant. (B) The total IgM concentration of the above supernatants as measured by ELISA. (C) LDA for the determination of frequencies of regulatory B cell clonal precursors in the spleen of newborn, young adult, and old animals. For each frequency determination, at least 6 different B cell concentrations were set up with 36 replicates per point. Negative standards were defined by a set of 36 cultures receiving only irradiated rat thymus feeder cells (3×10^5 /well) and LPS (50 $\mu\text{g/ml}$) but no murine B cells. After 6 days of incubation supernatants from LDA were assayed for the regulatory IgM as described in panel A. Supernatants showing a >35% inhibition of B cell proliferation were considered as positive. Data are representative of three similar experiments

competent to proliferate and secrete the autoantibody, but also that a significant part of them are already activated under physiological (in vivo) conditions, i.e., they belong into the actual repertoire of B lymphocytes [63]. This finding confirms the results of others [64] who observed that autoreactive B cells are often blast, cycling cells and they secrete autoantibodies into the natural immunoglobulin pool.

Accordingly, culture supernatants of LPS-stimulated B cells from the peritoneal cavity contained a somewhat higher amount of the regulatory IgM compared to that from the spleen. Lymph node B cells, on the other hand, did not produce the autoantibody in response to LPS (unpublished data).

Finally, cell depletion studies showed, that the regulatory cells are not only sIg, I-A, and B220 (CD45R) positive, but a significant part of them also bear the Ly-1 (CD5) glycoprotein (Table II). Therefore, we concluded that the regulatory B lymphocytes belong to the B1 cell subset. Their activation state, distribution in the body (peritoneal cavity > spleen >> lymph node), and cell surface marker phenotype (mainly Ly-1⁺) altogether suggest it [59–61].

Table II

The regulatory IgM mainly produced by Ly-1 (CD5)⁺ B cells^a

Reagents		³ H-thymidine incorporation (cpm/culture × 10 ⁻³ ± SD) ^b	Inhibition (%)
LPS	Culture supernatants produced by LPS stimulated spleen cells depleted for		
–	–	8.8 ± 1.3	
+	–	111.2 ± 22.1	
+	None	55.7 ± 4.8	50
+	Thy-1 ⁺ cells ^c	39.5 ± 5.2	64
+	Ly-1(CD5) ⁺ cells ^d	79.1 ± 6.6	29
+	sIg ⁺ cells ^e	101.9 ± 15.2	8
+	B220(CD45R) ⁺ cells ^e	96.6 ± 11.9	13
+	I-A ⁺ cells ^d	98.9 ± 7.2	11

^aDBA/2 spleen cells were cultured at 2 × 10⁵ cells/well in 200 µl final volume of complete medium either alone, or in the presence of LPS (10 µg/ml), or LPS plus B-SN (25% v/v) for 48 h. Proliferation was measured after 18 h additional incubation with ³H-thymidine

^bResults represent arithmetic mean ± SD of triplicate wells

^cDBA/2 spleen cells were depleted for T lymphocytes by monoclonal anti-Thy-1.2 antibody and complement

^dSpleen cells were separated into Ly-1⁺ and Ly-1⁻, or I-A⁺ and I-A⁻ populations by magnetobeads coated with monoclonal anti-Ly-1 or anti-I-A antibodies

^eSpleen cells were separated into sIg⁺ and sIg⁻, or B220⁺ and B220⁻ populations by panning on anti-µ or anti-B220 antibody coated plates

Implications for immunopathology: autoimmune diseases and immunodeficiencies

B cell hyperactivity is the general and cardinal feature of murine and human systemic lupus erythematosus (SLE), the prototype organ-non-specific autoimmune disease. The defect(s) responsible could be intrinsic to B cells, secondary to T cell or macrophage abnormalities resulting from excessive help or deficient suppression, or could stem from qualitative and/or quantitative abnormalities during the presentation of autoantigens [66, 67]. Thus, an important question is whether the abnormal production of, or the B cell response to, the regulatory IgM is associated with the development of murine SLE.

We found that splenic B cells of SLE-prone NZB mice responded much faster to LPS in terms of regulatory IgM production than normal mice [63]. This may reflect a higher percentage of spontaneously activated *in vivo* B lymphocytes in NZB mice as others have proposed [66]. In addition, LPS stimulated splenic B cells from young adult NZB mice proliferated more efficiently in the presence of the regulatory IgM than did B lymphocytes from age-matched normal or from old NZB animals [63]. The reason for this lower sensitivity of B cells from young adult NZB mice to the regulatory autoantibody is unclear at present. It could be caused by the inherent hyperresponsiveness of NZB B cells to accessory signals [68, 69] and/or their relative insensitivity to downregulatory effect(s) [70], and could contribute to the early pathogenesis of murine SLE. One could also speculate, however, that the composition of B cell population in NZB mice differs significantly from that of normal mice and largely relates to those B lymphocytes which mainly, but not uniformly express Ly-1. This subpopulation of B cells (B-1) has been found in increased frequency (5–10%) in spleens of young adult NZB mice as compared to normal animals (1–2%) [59].

Moreover, the spleens of old NZB mice have an abnormal (clonal or oligoclonal) population of B-1, hyperdiploid B cells which rapidly overgrow the normal, polyclonal B1 and B2 B lymphocytes and other spleen cells [71, 72]. Thus, a subset of LPS-reactive B lymphocytes uninhibitable by the regulatory IgM could disappear in NZB mice with aging.

Anti-lymphocyte – including anti-B cell – autoantibodies are also present in patients with SLE, but their primary involvement in the autoimmune process has received little experimental support [73, 74].

In the sera of most patients with Wiskott–Aldrich syndrome (WAS), a rare X-linked recessive immunodeficiency disorder, Brouet et al. [75] identified IgM autoantibodies that react with a subset of normal human peripheral and tonsillar B lymphocytes and induce terminal B cell differentiation without proliferation *in vitro*. Recently, two monoclonal anti-B cell autoantibody secreting hybridomas were generated from splenocytes of a patient with WAS. These antibodies stain several pre-B and B cell lines and do not react with most non-B cell lines. Moreover, they brightly stain >95% of the normal CD5⁺ B cells and less efficiently nearly all of the CD19⁺ lymphocytes. In contrast, B cells located within the germinal centre, that are engaged in secondary immune responses to antigens, are not stained by these monoclonal autoantibodies [76]. Therefore, it will be worth investigating a possible similarity, or even identity between the WAS autoantibodies and our regulatory IgM.

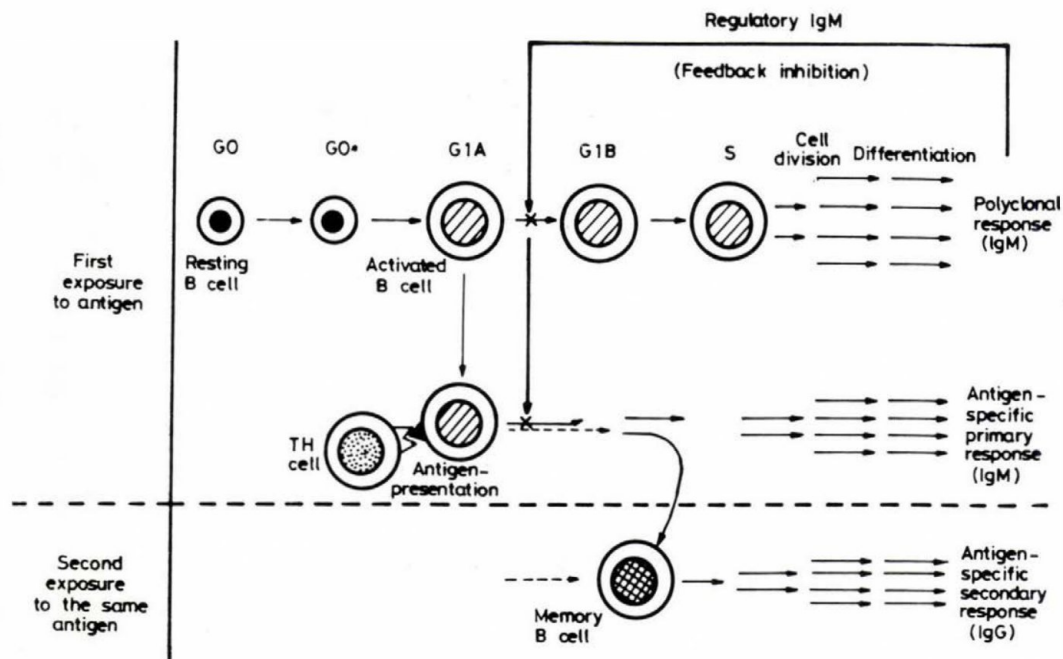


Fig. 3. Proposed model for the role of the regulatory IgM during B cell responses

Functional abnormalities of B cells in patients with AIDS and in mice with a leukemia virus (LP-BM5 MuLV) induced AIDS-like disease (MAIDS) were recognized soon after the epidemic appeared. The disease is characterized by a wide spectrum of immunologic abnormalities, including polyclonal activation of B cells, hypergammaglobulinaemia, and the production of autoantibodies, which are followed by severe deficiencies of B cell responses to polyclonal and antigen stimuli, and late in the disease, frequent occurrence of B lymphomas derived from the B1 cell subset [77, 78]. Numerous studies described anti-lymphocyte, including anti-B cell autoantibodies in the serum of patients during the progression of the disease [79, 80]. Accordingly, at least in the course of MAIDS an abnormal repertoire of antibodies is expressed, similar to the fetal repertoires known for degeneracy and autoreactivities, suggesting a relationship between the pathologic process and repertoire selection and giving additional support to the notion that retrovirus-induced immunodeficiencies might result from abnormal regulations and autoimmune dysfunction rather than direct cytopathic activation of the viruses [81]. The study of B cell deregulation and abnormal repertoire selection, however, seems marginal until now in comparison with the great mass of T cell oriented investigations. Thus, more studies are needed to clarify any possible role of anti-lymphocyte autoantibodies in AIDS.

Concluding remarks and speculations

In summary, we found that culture supernatants of LPS-stimulated murine B lymphocytes are able to inhibit the growth of freshly isolated normal B cells and neoplastic B cell lines via an IgM autoantibody. This B cell specific regulatory IgM allows the normal progression of early ($G0^+$, $G1A$) but not late ($G1B$, S , $G2$, and M) steps in the cycle of mitogen stimulated B lymphocytes. Conversely, it does not affect the increased antigen-presenting capacity of LPS-activated B cells. Furthermore, the autoantibody is able to inhibit polyclonal, as well as hapten-specific primary (IgM), but not secondary (mainly IgG1) antibody production, i.e. memory B cell responses (Fig. 3). Therefore, whereas newly emerging naive B cells are highly susceptible, memory B cells appear completely resistant to inactivation by the regulatory IgM autoantibody [32, 33, 41, 48, 54, 55, 63]. Taken together these data suggest a novel autoantibody-mediated pathway in the regulation of humoral immune responses, which allows the production of high affinity IgG, but not low affinity and often auto- and poly-reactive IgM antibodies in the antigen-specific responses of B lymphocytes.

May we count on a more general functional role of this regulatory IgM, such as an ability to conserve the immunological homunculus? We can confidently predict that this B cell specific natural autoantibody will on the whole be beneficial for our "little man". The immunological homunculus is an internal image of the self acquired by early recognition of self antigens, both in the thymus and in the periphery. The self image is, in fact, composed of the committees of autoreactive T and B cell clones that deal with the dominant self antigens. This type of autoreactivity is maintained at a low background level in normal individuals, under controls that prevent large-scale anti-self response [23, 24, 82–84]. Infection with a pathogen, however, could trigger a

response that includes expansion of the autoreactive population (by mimicry or polyclonal activation) along with the production of specific anti-pathogen antibody. Thus, elevated levels of autoantibodies may occur frequently in association with events such as infection [85, 86]. Moreover, polyclonal activation may also be an initiating event in autoimmune diseases [66]. In this case, how could the homunculus, which by definition is a delicate balance of regulated autoimmunity, survive the almost continuous pressure, i.e., how could the immune system keep our "little man" (and our body) healthy. In fact, the outcome in most individuals is that the autoreactivity is transient and does not become clinically apparent during immune responses. Therefore, the immune system seems to have created dams and sluices to mount attacks against the homunculus.

No doubt, connectivity of V-regions of antigen receptors (idiotypes) originally proposed by Jerne in his anti-idiotypic theory [87], a theory advanced by the work of many others [15, 20–22, 29, 88], can form a fine regulatory network inside the homunculus. Strong polyclonal activation during heavy bacterial infection, however, might easily brake this connectivity. In addition, several unconnected lymphocyte clones, cells from the peripheral immune system also become activated. As a result, our immune system will never again be an organized and regulated defense force. Thus polyclonal activation which cannot be avoided must be dealt with.

Fortunately, the immune system uses parallel regulatory pathways for safety and reliability. B cell specific natural IgM autoantibodies are able to prevent unlimited polyclonal B cell proliferation and differentiation [54]. Conversely, Wolf-Levin et al. [89] recently described a specific IgG autoantibody in normal human sera, which is a potent inhibitor of autoreactive T cell responses. Thus, the immune system seems to have evolved a mechanism to protect itself against an "all out" suicide attack.

We would like to propose that this autoantibody-mediated regulatory pathway is an integral part of the immunological homunculus. First, the murine B cell specific IgM inhibits both mouse, rat, and human B lymphocyte proliferation, i.e. the V-region of the regulatory autoantibody is probably germ-line encoded and conserved during evolution. It also means that the B cell surface structure recognized by the autoantibody cannot be very different in rodents and humans. So, it might be one of the "dominant autoantigens". Second, the regulatory IgM producing B cells belong into the B1 subset of lymphocytes which is, by participation in idiotypic networks, an important cellular basis of the homunculus. Finally, appearance and expansion of the regulatory B cell clones during ontogeny again suggests a highly specialized and conserved function for these B cells.

In conclusion, B cell specific natural IgM autoantibodies may constitute a novel important component of the regulation of humoral immune responses, in addition to other well-known autocrine/paracrine mechanisms such as downregulation by soluble immune complexes [90–92] and by inhibitory cytokines, for example TGF- β [93–94]. Furthermore, it could eventually lead to possible applications to the treatment of B cell neoplasias and autoimmune diseases, even before immune destruction of tissue or function has occurred.

Acknowledgments. We thank Drs László Kopper and Andrea Ladányi (1st Institute of Pathology and Experimental Cancer Research, Semmelweis University Medical School, Budapest) for kindly providing the HT-168.M1 and A549 human cell lines, and Drs George A. Füst and Anna Bíró for critical review of the manuscript. This work has been supported by the National Research Grant, OTKA T5323 and 190.

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GROWTH OF UNTREATED AND RADIATION-DAMAGED *LISTERIA* AS AFFECTED BY ENVIRONMENTAL FACTORS*

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(Received April 6, 1993)
(Accepted January 14, 1994)

The growth of untreated *Listeria monocytogenes* 4ab No. 10 and that of the surviving fraction of its population treated with 0.8 kGy gamma rays was investigated in a microtitre-plate system at incubation temperatures between 3 °C and 35 °C in Tryptic Phosphate Broth (TPB) or Brain Heart Infusion Broth (BHIB) media containing NaCl between 0.25 to 16.75% (w/v), and acidified with citrate-phosphate buffers to pH values between 4.63 and 7.06. The initial viable count was 3×10^3 /ml. Time periods to visible growth were recorded. Radiation survivors showed increased salt- and pH-sensitivities and increased minimum temperature for growth in TPB-based media. Adverse effects of sub-optimal environmental factors (reduced water activity, pH and temperature) and radiation injury were much less pronounced in BHIB-based media. Polynomial equations describing the combined effects of hydrogen ion and salt concentrations on the detectable growth at 30 °C were constructed for quantitative assessment of interactions. The results demonstrate that combining environmental stresses with low-dose irradiation can control growth of *L. monocytogenes*.

Various studies demonstrate that low doses of ionizing radiation which do not change the sensory quality and physical state of the product can extend considerably the shelf-life of specific refrigerated foods [1, 2]. It is important, however, that extended shelf-life, minimally processed chilled foods also maintain their microbiological safety with particular reference to those food-borne pathogens, which are capable of growth at refrigeration temperatures. *L. monocytogenes* grows at refrigeration temperatures, is enhanced by decreased oxygen and elevated carbon dioxide levels, and is tolerant of salt [3]. These characteristics motivated several previous studies on survival and growth of *L. monocytogenes* as affected by environmental factors [e.g. 4–6] which provided a background for our model investigations of the effect of some important environmental factors such as

*This paper was written in honour of the memory of Professor Zoltán Alföldy (1902–1992) director emeritus of the Institute of Microbiology Semmelweis University Medical School in Budapest (Hungary)

temperature, pH, salt-concentration (water activity) and nutrient media on growth of *L. monocytogenes* as influenced by a radurization dose.

Materials and methods

Organism. *L. monocytogenes* strain No. 10, serovar 4ab, obtained from Dr. B. Ralovich, (National Meat Research Institute, Budapest) was used as test organism. It was maintained on Brain Heart Infusion Agar (Oxoid CM 225 BHI-broth, with 1.5% Difco agar).

Radiation treatment. A stationary-phase culture of the test-organism grown at 30 °C for 24 h in Tryptic Phosphate Broth (TPB) [4] was harvested by centrifugation at 10 000 rpm for 20 min and aseptically re-suspended in sterile "physiological" phosphate buffer (pH 7.0) containing 0.1% peptone. The buffered suspension was irradiated with various doses of gamma rays by a self-shielded ^{60}Co -irradiator (type RH-30) at the Central Food Research Institute, Budapest, to estimate the D_{10} -value. Surviving fractions were determined by plate counting in TP-agar with incubation at 30 °C for 5 days. A D_{10} value of 0.4 kGy was calculated from the semilogarithmic linear survival curve. For the growth experiments cell suspensions prepared as above were irradiated at 0.8 kGy radiation dose.

Growth experiments. Sterile, 96-well microtitre-plates were used to assess the effects of combinations of environmental factors on growth of untreated or irradiated inocula.

In the first experimental series, twelve concentrations of NaCl (0.25–16.75% w/v, in approximately equal increments) were combined aseptically with eight hydrogen ion concentrations in approximately equal increments from 0.1 $\mu\text{mol/l}$ (pH=7.06) to 6.5 $\mu\text{mol/l}$ (pH=5.17) in TPB. Hydrogen ion concentrations were adjusted with mixtures of citric acid (0.1 mol/l) sodium-hydrogen orthophosphate (0.2 mol/l) buffers [7]. Sterilized citrate/phosphate buffers inoculated with unirradiated or irradiated populations of the test organism were combined aseptically in 1:1 v/v ratio with sterile TPB media containing different salt concentrations to give the final desired concentrations of hydrogen ions and NaCl. Thus, the culture media were half the strength of the concentrations in the original nutrient media. Taking into account that the 0.8 kGy gamma radiation dose resulted in a 99%-destruction of the test organism, inoculum levels were adjusted such way that the initial viable cell count in the wells was $3 \times 10^3/\text{ml}$, irrespective of whether or not the inoculum had been irradiated. Inoculated plates containing 250 μl culture in each well were aseptically sealed with self-adhesive COSTAR foils. Duplicate plates were incubated at 35, 30, 10 and 5 °C. Wells were checked visually for bacterial growth daily during the first 10 days of incubation, thereafter every three days for up to 10 weeks, and time periods to visible growth were recorded for each well. It was estimated that "visual growth" represents an increase of at least 1000-fold in the cell counts per ml [8]. Samples from selected wells that showed growth were plated on BHI-agar to check for purity.

A similar plan was used for the second experimental series, except that Brain Heart Infusion Broth (BHIB) was used as the basal medium, the pH-range was between pH 7.06 ($[\text{H}^+]$ 0.1 $\mu\text{mol/l}$) and 4.63 ($[\text{H}^+]$ 28 $\mu\text{mol/l}$) in twelve increments, and the salt concentration were between 0.25% and 14.25% in eight increments. Incubation temperatures in the second experimental series were 30, 20, 8 and 3 °C.

Mathematical model. For 30 °C incubation temperature only, the reciprocal of the natural log of times to growth (100/TG) (day^{-1}) were taken as "apparent growth rates" and analysed by a least squares fit to a quadratic model and stepwise variable selection by a Statgraphics software on a personal computer. The concentration of salt [S] (%) in the media and the hydrogen ion concentration $[\text{H}^+]$ ($\mu\text{mol/l}$) were used as independent variables in the construction of the model equations. It is known from the paper of Cole et al. [6] that conversion of pH values into hydrogen ion concentrations may result in a more simple equation. The above growth term was expressed as natural log on the basis of consideration by Cole et al. [6], assuming that the observed changes in "apparent growth rate" are mainly influenced by the changes in the logarithmic period, which increases exponentially as the limits for growth are approached [9, 10]. The polynomial functions calculated were used to construct three-dimensional surface plots to visualise the relationship of the growth variable to the environmental parameters.

Results and discussion

The effects of various factors on the time to visible growth within a 71-day incubation period are shown in Tables I and II for the first and second experimental series. Growth data are summarized at selected pH values for the eight salt concentration levels in Table I, and for the first seven salt concentration levels in Table II because no visible growth occurred during 10 weeks of incubation at high salt concentrations which are omitted from the tables.

Table I

Effect of pH and salt concentration on the growth of untreated and irradiated L. monocytogenes in TPB

pH/[H ⁺] μmol/l	NaCl %	Time to visible growth (days) in duplicate microtitre plates															
		Unirradiated								0.8 kGy							
		35 °C		30 °C		10 °C		5 °C		35 °C		30 °C		10 °C		5 °C	
7.07/0.1	0.25	2	2	2	2	20	20	64	64	2	2	2	2	20	20	64	64
	1.75	2	2	2	2	20	20	64	64	2	2	2	2	20	20	64	64
	3.25	2	2	2	2	20	20	64	64	2	2	2	2	24	24	64	—
	4.75	2	2	2	2	24	24	—	—	3	3	3	3	24	24	—	—
	6.25	3	3	6	6	24	24	—	—	—	—	6	6	31	31	—	—
	7.75	—	—	6	6	24	24	—	—	—	—	6	6	52	—	—	—
	9.25	—	—	13	13	71	41	—	—	—	—	—	—	—	—	—	—
	10.75	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5.90/1.26	0.25	2	2	3	3	24	24	64	64	3	3	3	3	31	34	64	—
	1.75	6	6	3	3	34	34	—	—	—	—	24	—	—	41	—	—
	3.25	8	—	6	8	34	34	—	—	—	—	24	—	—	—	—	—
	4.75	—	—	20	20	71	64	—	—	—	—	—	—	—	—	—	—
	6.25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5.53/2.95	0.25	6	6	6	6	64	64	—	—	7	6	7	7	—	—	—	—
	1.75	7	8	7	6	—	71	—	—	—	—	—	—	—	—	—	—
	3.25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5.33/4.68	0.25	6	6	6	6	—	—	—	—	34	7	13	24	—	—	—	—
	1.75	—	24	13	13	—	—	—	—	—	—	—	—	—	—	—	—
	3.25	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5.17/6.46	0.25	7	8	7	7	—	—	—	—	—	—	—	24	—	—	—	—
	1.75	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Table II

Effect of pH and salt concentration on the growth of untreated and irradiated L. monocytogenes in BHIB

pH/[H ⁺] μmol/l	NaCl %	Time to visible growth (days) in duplicate microtitre plates															
		Unirradiated								0.8 kGy							
		35 °C		30 °C		10 °C		5 °C		35 °C		30 °C		10 °C		5 °C	
7.06/0.1	0.25	1	1	2	2	6	6	27	27	1	1	2	2	6	6	31	31
	2.25	1	1	2	2	6	6	27	27	1	1	2	2	8	7	34	31
	4.25	1	1	2	2	7	8	27	27	2	2	2	2	8	8	36	36
	6.25	2	2	3	3	10	10	36	36	2	2	3	3	13	13	48	43
	8.25	3	3	6	6	17	17	–	–	3	3	6	6	20	20	–	–
	10.25	17	–	7	7	41	41	–	–	–	–	7	7	48	48	–	–
	12.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5.61/2.5	0.25	1	1	2	2	8	8	–	–	1	1	2	2	13	10	–	–
	2.25	1	1	2	2	17	17	–	–	2	2	3	3	22	20	–	–
	4.25	2	2	3	3	–	–	–	–	2	2	6	6	24	–	–	–
	6.25	3	3	6	6	–	–	–	–	3	3	7	7	–	–	–	–
	8.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5.25/5.6	0.25	1	1	2	2	27	27	–	–	2	2	3	3	24	24	–	–
	2.25	2	2	3	2	–	–	–	–	2	2	6	6	45	–	–	–
	4.25	3	3	6	6	–	–	–	–	3	3	7	7	–	–	–	–
	6.25	–	–	29	22	–	–	–	–	7	7	17	15	–	–	–	–
	8.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5.12/7.6	0.25	2	2	3	3	44	–	–	–	2	2	6	6	–	–	–	–
	2.25	2	2	6	6	–	–	–	–	2	2	6	6	–	–	–	–
	4.25	3	3	8	8	–	–	–	–	6	6	13	10	–	–	–	–
	6.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4.89/12.8	0.25	3	3	–	–	–	–	–	–	6	6	–	–	–	–	–	–
	2.25	3	3	22	24	–	–	–	–	6	6	34	31	–	–	–	–
	4.25	–	–	29	29	–	–	–	–	–	–	43	34	–	–	–	–
	6.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4.72/19.1	0.25	8	9	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2.25	22	22	–	55	–	–	–	–	34	22	–	–	–	–	–	–
	4.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4.63/23.4	0.25	17	24	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	2.25	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

– no growth within 10 weeks

It can be seen from Table I that pH- and salt-sensitivity of growth of the test organism depended considerably on the incubation temperature. Sensitivity to salt and pH were higher the further the incubation temperature was from the optimum 30 °C. Adverse environmental factors (suboptimal water activities, pH and temperatures) and radiation injury both caused changes in growth limits and decreased the apparent growth rate. After 10 weeks of incubation, no survivors could be subcultured from

those wells where no visible growth was observed. At any given pH-level, the temperature and salt combinations permitting visible growth were slightly narrower when irradiated cells were compared to unirradiated. Similarly, the growth of radiation survivors was inhibited at somewhat lower salt concentrations and higher pH-values than that of the unirradiated bacteria. There was little change in salt sensitivity but a substantial change in pH sensitivity. The adverse effect of radiation injury and suboptimal environmental factors on growth expressed itself even within conditions permitting growth as evidenced by increased time periods for visible growth. It is worth noting that near the limiting values for growth, the replicate values became increasingly erratic.

In the second experimental series using BHIB, which is a more complete nutrient medium than TPB, the pH-minima were considerably lower and the salt-tolerance levels were higher than in those observed in TPB series.

For quantitative analysis of interaction of environmental factors and the effect of radiation injury on growth, the data obtained at 30 °C, at which most information was obtained, were analysed statistically. After omitting terms insignificant at 95% probability level, the resulted polynomial equations had the form of

$$\ln 100/TG = a - b[H^+] - c[S]^2 - d[H^+][S]$$

Table III summarizes the constants of the quadratic models and determination coefficients (r^2) for the four combinations of radiation dose and media.

Table III

Constants of the quadratic model $\ln (100/TG) = a - b[H^+] - c[S]^2 - d[H^+][S]$ and the determination coefficients (r^2) for the four combinations of radiation dose and media

Media	Dose (kGy)	a	b	c	d	r^2
TPB	0	3.91	0.166	0.020	0.157	0.751
	0.8	3.99	0.295	0.013	0.577	0.890
BHIB	0	5.05	0.133	0.028	0.010	0.907
	0.8	4.81	0.157	0.027	0.006	0.914

The equations show that the apparent growth rate was significantly higher in BHIB-based media than in TPB-based media. The logarithm of the apparent growth rate was a linear function of the hydrogen ion concentration and a quadratic function of the salt concentration. In TPB-based media a significant interaction (synergism) was found between the effects of hydrogen ion concentration and salt concentration. The sensitizing effect of radiation injury resulted in considerably increased constants for the $[H^+]$ and $[H^+][S]$ terms. However, no statistically significant radiation sensitization effects were indicated with the more rich (BHIB-based) growth medium.

To illustrate the interactions, three-dimensional response surfaces corresponding to the quadratic models are shown in Fig. 1.

The roles of pH, salt concentration and radiation injury as well as their combined effects on the growth kinetics at 30 °C are also illustrated by the positions of the "growth/no growth" boundary lines for various incubation periods as shown in Fig. 2.

Figure 2 shows well that the combined effects of sub-optimal environmental factors and radiation injury depend on the nature of media.

The observed effects of media and incubation temperature agree with other observations that effects of inhibitory factors on stressed cells are markedly influenced by nutrient composition and conditions of incubation [11]. Our observation that the variability of data tended to increase the further the conditions deviated from optimal are also known from other reports [6, 12, 26]. In such cases ln-transformation helps to stabilize the variance [10, 12].

Our observations that low pH values and high salt concentrations that did not permit growth inactivated *L. monocytogenes* on prolonged incubation is also in agreement with others [6]. Interestingly, in BHIB-based media at 20 °C, our test organism was growing at lower pH values when the salt concentration was higher (2.25% and 4.25%) than that of the original media (0.25%) (see Table II). This underlines the observation of Cole et al. [6] that low concentrations of salt provided a slight protective effect against inactivation of *L. monocytogenes* at low pH values. The lowest pH 4.63 we applied with half-strength BHIB acidified with citric acid permitted slow growth, but only with the lowest salt concentration, and only at the optimum growth temperature. Under similar conditions Cole et al. [6] noted slow growth of *L. monocytogenes* ATCC 19115 at pH 4.66 and up to 8% salt concentration, but no growth occurred at pH 4.53. The ability of *L. monocytogenes* to grow at low pH values is obviously greatly influenced by the nature of the acidulant. In an enriched trypticase soy broth acidified by HCl the minimum pH permitting growth was 4.4 at 30 °C and 20 °C, but pH 5.2 at 4 °C [5]. In other studies with unclarified cabbage juice acidified with lactic acid no growth occurred at pH 4.8 [4]. The minimum pH value required for the initiation of growth of *L. monocytogenes* in BHIB ranged from 4.3 to 5.2 at 30 °C, and 5.0 to 5.7 at 4.0 °C, depending upon the acidulant used [3]. These observations are in agreement with the general understanding that at the same pH values organic acids are more inhibitory to microorganisms than inorganic acids [14] because they have additional inhibitory effects besides their effect on the pH [15]. Razavilar and Genigeorgis [16] reported recently that depending on temperature the minimum pH supporting growth for *L. monocytogenes* in BHIB in presence of a specific acid was: 4.3–4.8 for hydrochloric acid, 4.4–4.9 for citric acid, 4.6–5.1 for lactic acid, 5.0–5.2 for acetic acid, and 5.5–5.95 for propionic acid. Our data is consistent with observation of other authors [5, 6, 8, 12, 16] that the ability of *L. monocytogenes* to grow at low pH levels is highly dependent on incubation temperature.

Our test organism showed a limiting salt concentration level between 9.25% and 10.75% in the half-strength TPB and between 10.25% and 12.75% in the half-strength BHIB at optimal pH and temperature. Thus, our strain seemed to be less salt tolerant than *L. monocytogenes* ATCC 19115 investigated by Cole et al. [6], who observed growth in TPB containing 12% salt added.

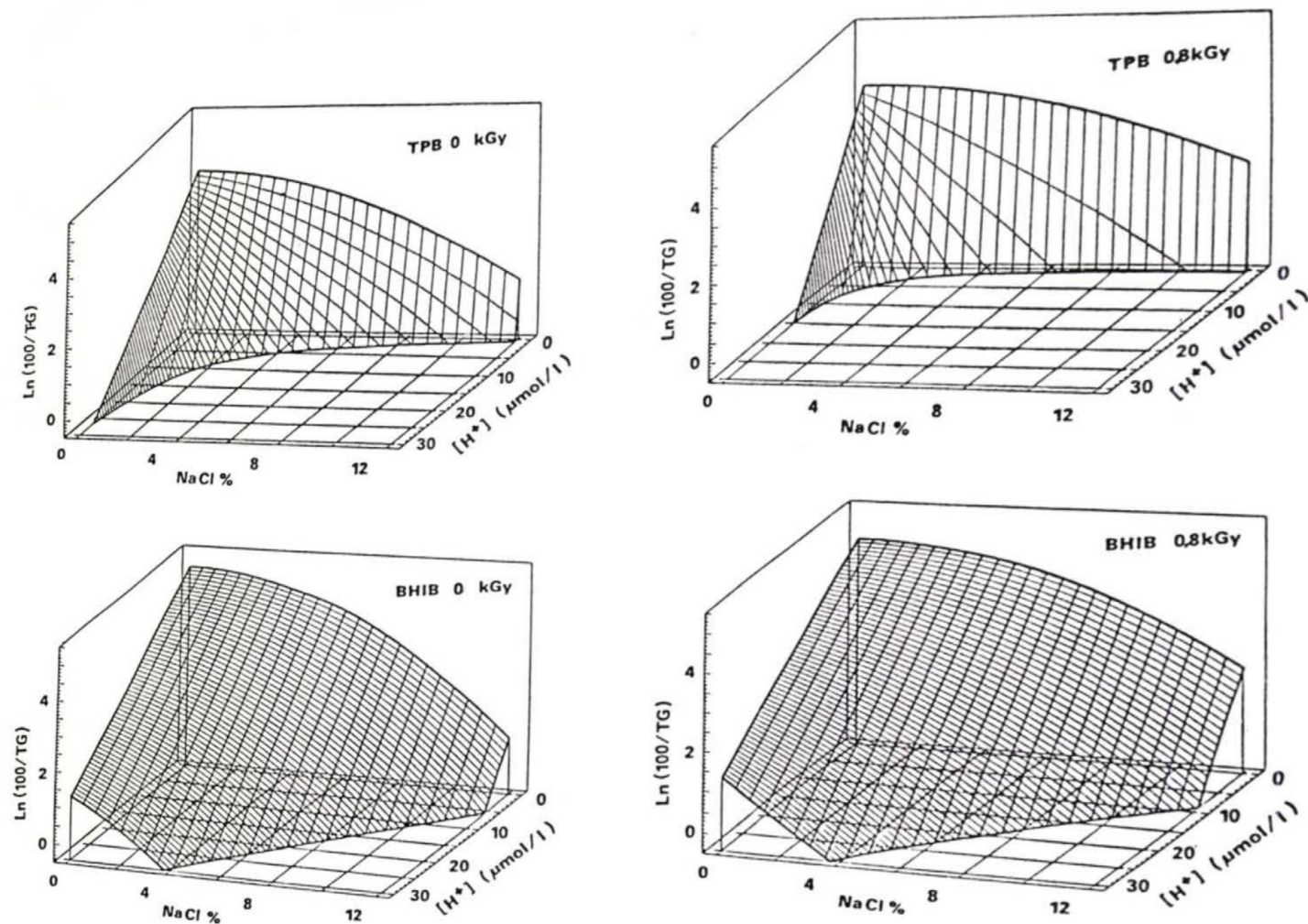


Fig. 1. Response surfaces corresponding to the polynomial equations describing the combined effect of hydrogen ion and salt concentrations on the apparent growth of untreated (0 kGy) and irradiated (0.8 kGy) populations of *L. monocytogenes*. TG = time to visually observable growth in days; TPB = Tryptic Phosphate Broth; BHIB = Brain Heart Infusion Broth

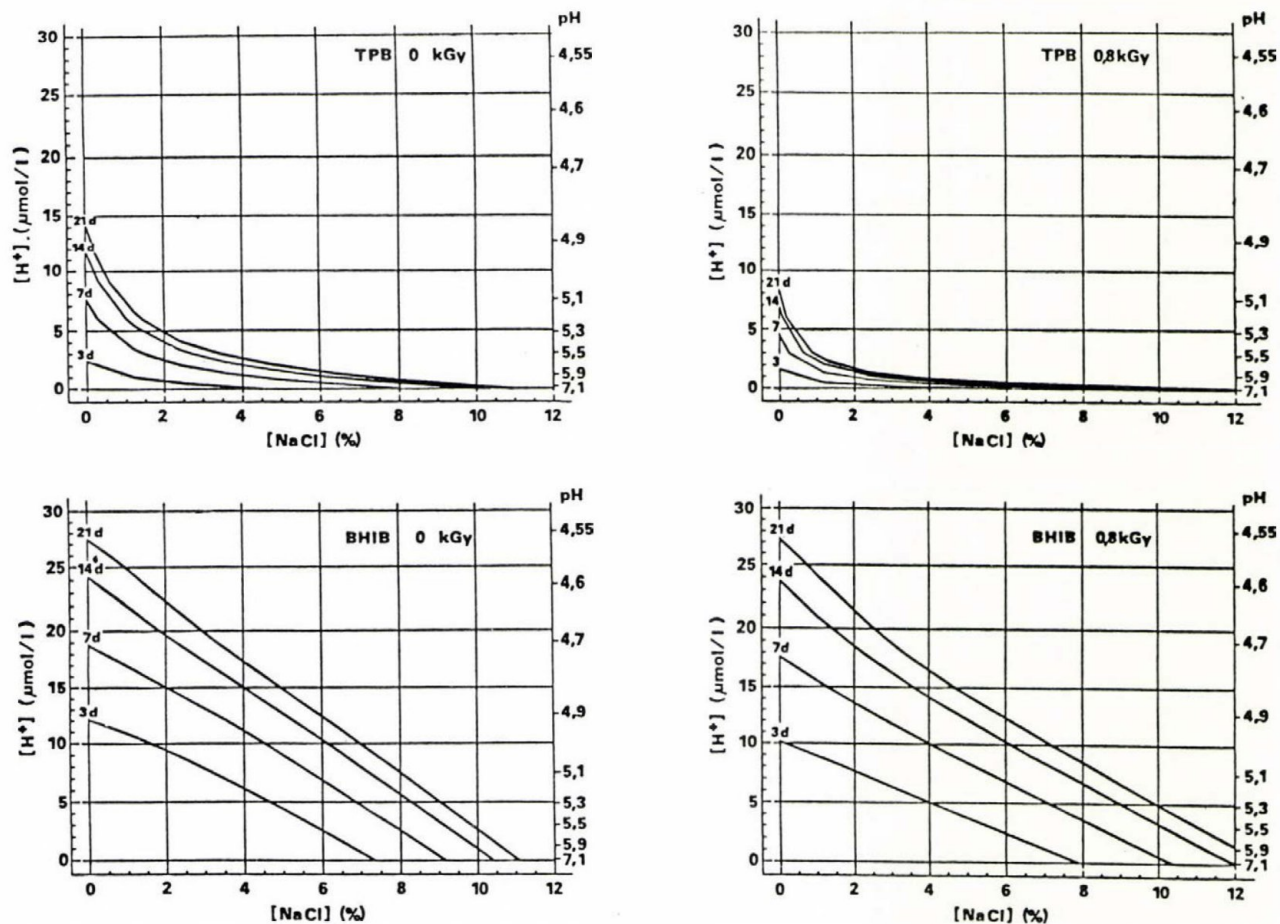


Fig. 2. Border-lines of growth/no growth of untreated (0 kGy) and irradiated (0.8 kGy) populations during 3, 7, 14 or 21 days of incubation at 30 °C.
TPB = Typtic Phosphate Broth; BHIB = Brain Heart Infusion Broth

However, Conner et al. [4] reported that *L. monocytogenes* (Scott A and LCDC 81-861) grew in Tryptose Soy Broth (TSB) (pH 7.3) at 30 °C in the presence of 10% NaCl but not at 12% within two weeks. *L. monocytogenes* NCTC 9863 investigated by McClure et al. [8] was growing e.g. even at 8% NaCl-level, pH 5.0 and 20 °C in TSB. Nevertheless, the lower salt tolerance in our model systems might also be related to lower concentrations of certain osmoprotectants that are probably present in rich media. Recent studies of Te Giffel et al. [17] showed that *Listeria* is able to grow in the presence of higher salt concentrations in a rich medium (BHIB) than in a chemically defined minimal medium developed for *L. monocytogenes* by Premaratne et al. [18].

The polynomial equation, which describes the effect of hydrogen ion concentration on growth in a linear term, and the effect of salt concentration as a quadratic relationship is consistent with the mathematical model constructed by Cole et al. [6]. As pointed out by them, the quadratic relationship found to describe the effect of salt concentrations indicates that there may be an optimum salt concentration for survival and growth of *L. monocytogenes*. Contrary, however, to the findings of Cole et al. [6], a significant interaction (synergism) between the salt concentration and hydrogen ion concentration on the growth of *L. monocytogenes* was found in our experiments, particularly with TPB-based media and radiation-damaged populations.

Our test organism with its D_{10} value of 0.42 kGy in pH 7.0 "physiological" phosphate buffer seems to be slightly more radiation resistant than the strain investigated by Stegeman [19] who obtained D_{10} values of 0.2 and 0.38 kGy in buffer and minced meat (both at 4 °C), respectively, and that used by Tarján [20] who reported that irradiation of *L. monocytogenes* with 2 kGy in culture media or pork meat paste resulted in a 10^7 reduction in viable counts. On the other hand, D_{10} values of 0.42–0.55 kGy in poultry meat [21], and 0.34–0.5 kGy in TSB and 0.51–1.0 kGy in raw ground beef [22] have been reported depending on the strain and recovery media.

Our results on the increased susceptibility of irradiated population surviving a 2 D_{10} radiation dose indicate a sublethal injury of the radiation survivors. This is consistent with the findings of Patterson [21], Mead et al. [23] and Patterson et al. [24] who reported an increase of the lag phase of any *L. monocytogenes* which survived an irradiation process. For example, in cooked poultry stored at 6 °C, the lag duration of *L. monocytogenes* was 1 day in unirradiated samples and 18 days in samples that had been given a 2.5 kGy irradiation treatment [24]. Also Areal et al. [25] reported recently decreased apparent D_{10} values by direct plating irradiated suspensions of *L. monocytogenes* on different selective solid media.

The results of our model studies demonstrate the efficiency of combining environmental stresses. Low-dose irradiation in combination with mild antimicrobial factors can be manipulated to contribute to the microbiological safety of such extended shelf-life chilled products where *L. monocytogenes* presents a potential hazard.

Acknowledgements. This study was supported by the Hungarian Scientific Research Fund (OTKA) Project No. 611. and in part by the International Atomic Energy Agency Research Contract No. 6271/497. The authors thank Dr. R. L. Buchanan and Dr. T. A. Roberts for valuable advice and discussions.

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PRODUCTION AND SOME CHARACTERISTICS OF β -GLUCOSIDASE IN *DIAPORTHE* (*PHOMOPSIS*) *HELIANTHI*

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(Received July 14, 1993)
(Accepted January 14, 1994)

Diaporthe (Phomopsis) helianthi Munt.-Cvet. et al. is an important phytopathogenic fungus which causes stem canker of sunflower. When grown in submerged cultures in the presence of milled wheat chaff as a carbon source this phytopathogen produced extracellular and intracellular β -glucosidases. The optimum pH of these enzymes was 4.8. The temperature of crude intracellular β -glucosidase activity was at 60 °C, whereas the optimum activity of crude extracellular β -glucosidase was observed in a wide range of temperature between 40 and 70 °C. Although, extracellular and intracellular β -glucosidase activities had identical pH and similar temperature optima, the thermal stability of the intracellular enzyme was significantly higher.

The β -glucosidases have been studied for their importance in nature as carbon recycling enzymes, their potential in industrial conversion of lignocellulosic wastes to fermentable sugars [1, 2] and their phytopathogenic potential in the degradation of cell wall of plants [3].

Microbial degradation of cellulose in plant cell walls requires the synergistic action of β -1,4-endoglucanase (EC 3.2.1.4) which cleaves the polymer in a random fashion to release free chain ends, and β -1,4-exoglucanase (EC 3.2.1.91) which cleaves cellobiose units from the non-reducing chain ends; β -glucosidase (EC 3.2.1.21) converts cellobiose to glucose [4].

β -Glucosidase is not only a cellobiose-cleaving enzyme yielding glucose as its sole hydrolysis product, but it also acts on arylglycosides by cleaving aryl groups from the glycoside.

Diaporthe (Phomopsis) helianthi Munt.-Cvet. et al. is a new causative agent [5, 6] whose mechanism of pathogenesis is not sufficiently known. It has been described,

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however, that most plant pathogens produce enzymes that degrade components of cell walls of plants (cell-wall degrading enzymes – CWDE) [7–9]. As far as we know, there are no reports about the capability of this fungus to produce enzymes belonging to CWDE, except of pectinases [10].

The aim of this work was to study the ability of this phytopathogenic fungus to produce extra- and intracellular β -glucosidases in submerged cultures and to determine some of their characteristics.

Materials and methods

Microorganism. *Diaporthe (Phomopsis) helianthi* Munt.-Cvet. et al. was kindly supplied by Dr. M. Mihaljčević. This microorganism was isolated from the infected sunflower plants grown on the fields of Vojvodina (Yugoslavia), and maintained on potato-dextrose-agar (PDA) in Petri dishes.

Substrate. The starting material was the light fractions separated from wheat by air stream on a laboratory grain cleaner (Falling number AB type 51150, Sweden). The chaff thus obtained was ground on a laboratory mill (Christy and Norms Ltd, Chelmsford, UK, size 8 inch) fitted with a sieve (13 holes per inch, each hole 1 mm in diameter). Chemical composition of this substrate, determined by AOAC methods [11] is shown in Table I.

Fermentation medium. The medium used was that developed by Reese and Mandels [12]. The basal medium consisted of: 1% substrate (milled chaff as main carbon source), KH_2PO_4 1.5 g/l, $\text{MgSO}_4 \cdot \text{H}_2\text{O}$ 0.4 g/l, NaNO_3 1 g/l; CaCl_2 0.2 g/l, peptone 0.1 g/l, Tween 80 1 ml/l, trace element solution, 1 ml/l. The trace element solution contained (per litre): $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 5 g; MgSO_4 , 1 g, CoCl_2 , 1 g and ZnCl_2 1 g. The pH was adjusted to 5.0. Sterilization was carried out at 121 °C and 15 psi.

Preparation of inoculum. The inoculum was prepared in the following manner. A PDA dish was inoculated with a micelic disc (0.89 cm²) and incubated at 30 °C for 7 days, which was sufficient to obtain well-developed micelia. Ten ml of sterile distilled water were added to the dish, and after mild shaking withdrawn. This micelial suspension was used to inoculate (8 ml suspension/100 ml medium) preculture flasks containing Reese and Mandels [12] medium with 1% milled chaff as carbon source (see above). After 7 days of growing in the medium at 30 °C in a shaker (200 rpm), this preculture was used for inoculation of the fermentation medium (10% v/v).

Fermentation conditions. All fermentations were carried out in 300 ml shake flasks with a final working volume of 100 ml. Shake flasks were incubated in orbital shaker (200 rpm) at 30 °C. In order to avoid difficulties in the withdrawal of samples during the process, a sufficient number of flasks was used and the total content of two flasks was taken for evaluation at the desired time intervals.

Enzyme preparation. The mycelium was harvested at different fermentation times by centrifuging the fermentation broth (6000 g, 20 min, 4 °C) and the supernatant was used for the determination of extracellular enzyme activity. Phenylmethylsulphonyl fluoride (PMFS) was added to the supernatant at final concentration of 0.025 mmol/l to inhibit protease activity.

The pellets were washed with 0.05 mol/l sodium acetate buffer pH 4.8 and used for preparation of the cell-free extract from *P. helianthi*. Several methods were applied to prepare cell-free extracts [13–15] and the most effective was disruption of mycelium with osmotic shock [15].

The pelleted mycelium was suspended in 10 mmol/l acetate buffer pH 5.0 with 0.025 mmol/l PMFS for 24 h with stirring at 4 °C and centrifuged (17000 g, 30 min). The crude extract was centrifuged again as described. above. Samples were examined microscopically to confirm that most of the hyphae had been disrupted: the cell-free extracts were then assayed for β -glucosidase activity.

Enzyme assays. β -Glucosidase activity was measured according to the method of Wood [16], as aryl- β -glucosidase activity, by the hydrolysis of p-nitrophenyl- β -D-glucopyranoside. The concentration of p-nitrophenol was determined from the absorbance at 410 nm under alkaline

conditions, induced by 2 mol/l Na_2CO_3 . Units were defined as the amount of the enzyme required to release 1 μmol p-nitrophenol per hour.

To determine temperature optima, the activities were measured by incubation in the range 20–70 °C.

The determination of pH optima was carried out at the optimal temperature of the respective enzyme. Buffers (pH 4.0 to 7.0) were prepared by mixing 0.2 mol/l Na_2HPO_4 and 0.1 mol/l citric acid.

Enzyme stability was determined by exposing the crude enzyme to a given temperature in a water bath before samples were withdrawn at different time intervals, and residual enzyme activity determined. The determination of enzyme stability was performed at the optimal temperature and optimal pH of the respective enzyme.

Analytical methods. The content of reducing groups was determined by the DNS-method [17]. Biomass production was measured as a crude protein content. After centrifugation, the pellet was washed with 0.1 mol/l NaCl and dried at 100 °C to constant weight, and analyzed for total nitrogen (Nx) using the Kjeldhal method (AOCA 1975) [11], thus yielding the crude protein in the solid as $\text{Nx} \times 6.25$.

Protein content was measured by the Lowry et al. [18] method using bovine serum albumin as a standard.

Results

The fungus *P. helianthi* exhibited a significant growth in a liquid medium in the presence of ground chaff as source of carbon and produced β -glucosidase activity (Figs 1 and 2). As can be seen, the maximum of biomass production was achieved 100 h after inoculation, and this level remained during the next 90 h (Fig. 1B). In the first 40 h, the fungus used for its growth the fermentable sugars and soluble proteins, which resulted in a substantial decrease of the concentrations of these compounds in the culture supernatant (Fig. 1A).

Table I

Chemical composition of substrate (all values expressed as percent of dry weight)

Constituent	Value
Dry weight	91.4
Crude protein ($\text{N} \times 6.25$)	12.21
Soluble sugar	4.20
Cellulose	18.48
Ash	5.94
Starch	25.83

By utilizing these compounds, the fungus changes pH of the medium, causing its acidification (pH decreased from 5.4 to 4.8). When the medium is exhausted, i.e. when the easy-degradable sugars are used up (which is reflected in an abrupt decrease in the concentration of reducing sugars from 0.58 to 0.09 mg/ml), the fungus starts to

consume the more complex compounds, such as cellulose. In this stage the fungus does not use soluble proteins any more, but on the contrary, it starts to secrete extracellular proteins (Fig. 1A).

Under these cultivation conditions, the fungus produces both intra- and extracellular β -glucosidase (Fig. 2). The maximum of extracellular activity of this enzyme is observed in the stationary phase of the fungus growth, right then when the concentration of extracellular proteins is high and when the concentration of reducing sugars in the external medium is low (0.09 ng/ml). By comparing the curve for the fungus growth and the curve for the production of extracellular enzyme secretion is not associated with the fungus growth.

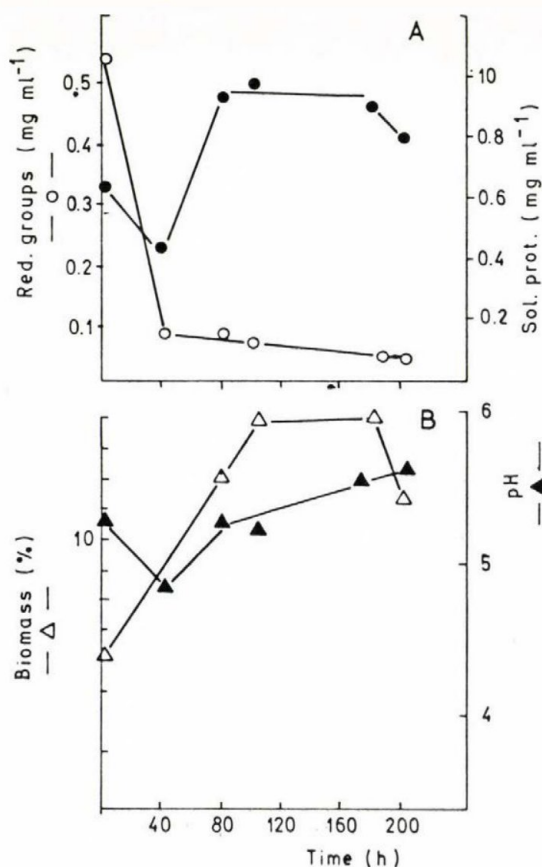


Fig. 1. Time course of mycellar biomass, reducing sugar and extracellular soluble protein content and pH during submerged fermentation of *P. helianthi*. Fungus was grown in 1% milled chaff as a carbon source at 30 °C, with initial reducing groups content of 0.56 mg/ml

The obtained results show that the time course of production of intra- and extracellular β -glucosidase during the growth of *P. helianthi* are different. The maximum activity of the intracellular enzyme lags behind the one for the extracellular enzyme about 120 h.

In Figs 3–5 are illustrated the properties of the investigated β -glucosidase from the fungus *P. helianthi*, such as the effect of pH, temperature, and the enzyme stability.

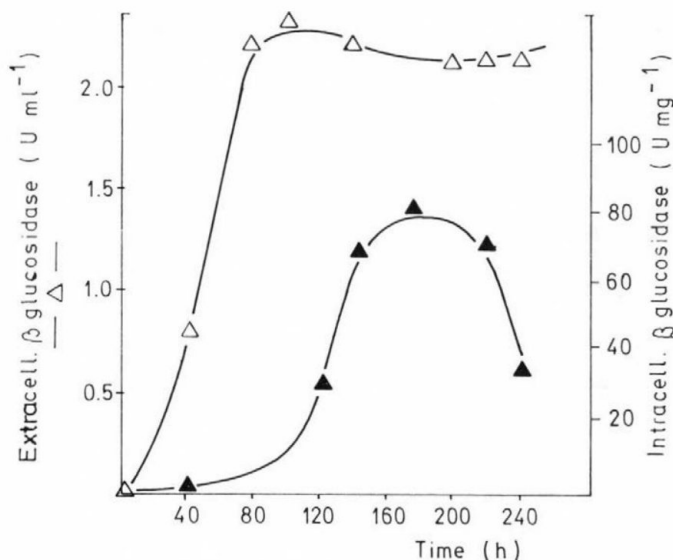


Fig. 2. Time course of production of extra- and intracellular β -glucosidase during cultivation in shake flasks with initial reducing groups content 0.56 mg/ml $\blacktriangle$ activity of intracellular β -glucosidase; $\triangle$ extracellular β -glucosidase

Though the time course of production of intra- and extracellular β -glucosidase are significantly different (Fig. 2), both enzyme types exhibited an identical pH optimum of 4.8 (Fig. 3), and a similar temperature optimum (Fig. 4). The only significant difference was observed in their thermal stability (Fig. 5). Approximately 80% of the extracellular activity was lost after 1 h of incubation at 60 °C, while under the same incubation conditions, the activity of intracellular enzyme was reduced only 15%.

Discussion

The results presented show that the fungus *P. helianthi* can produce extra- and intracellular β -glucosidase under the submerged conditions of fermentation (Fig. 2).

The levels of β -glucosidase activity in *P. helianthi* appear to be similar to those found in other fungi producing nitre- and extracellular β -glucosidase [14, 19–23].

The different time-course and extent of nitre- and extracellular enzyme production indicate that β -glucosidase is released from the cell. A low intracellular β -glucosidase activity compared with the high extracellular activity in the first 120 h of inoculation, shows that β -glucosidase is apparently released directly into the culture fluid. This mechanism has also been found in some other fungi [14, 19, 23].

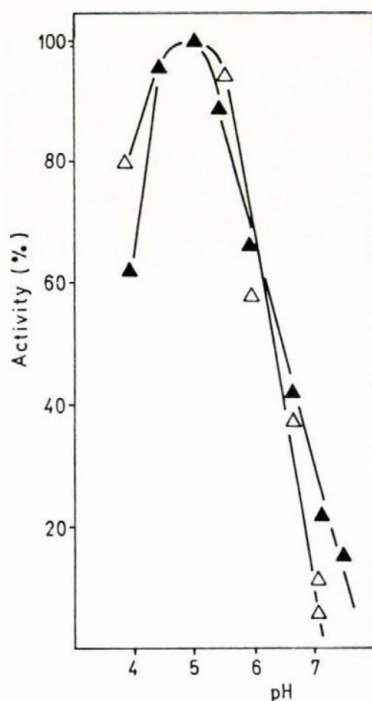


Fig. 3. pH optima of intra- and extracellular β -glucosidase of *P. helianthi*. Initial rates of the enzyme catalyzed reaction were measured. Reaction mixture was buffered with 0.2 mol/l Na_2HPO_4 and 0.1 mol/l citric acid and incubated at 50 °C. ▲ intracellular β -glucosidase activity; △ extracellular β -glucosidase activity

Although extra- and intracellular β -glucosidase activities had identical pH and similar temperature optima, the thermal stability of the intracellular enzyme was higher (Fig. 5). When found in the cultural fluid, the thermal stability of β -glucosidase is considerably lowered, possibly because this stability may be due to the intracellular factors [21]. The biphasic nature of the thermal inactivation curve of extracellular β -glucosidase suggests the presence of two extracellular β -glucosidase forms in this fungi.

The role of microbial β -glucosidase in pathogenesis was studied for a large number of plant diseases caused by different microorganisms [3, 24–26]. The conjugated phenols, which are the substrates for β -glucosidase, could become available

following the action of CWD enzymes on the cell walls of the vascular elements in the xylem [7].

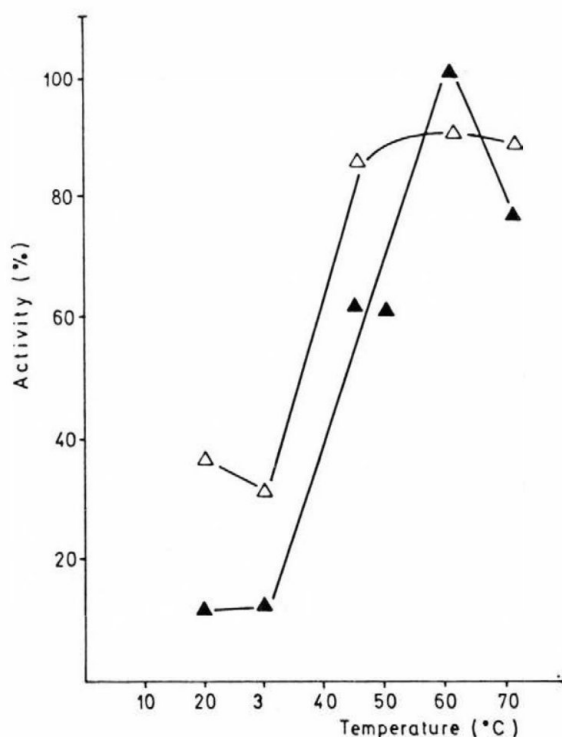


Fig. 4. Temperature dependence of extra- and intracellular β -glucosidase activity from *P. helianthi*. Initial rates of enzyme catalyzed reaction were measured at pH 4.8; $\blacktriangle$ intracellular β -glucosidase activity; $\triangle$ extracellular β -glucosidase activity

According to the histological studies by Muntanola-Cvetković et al [5, 6], after the penetration of *P. helianthi* into the sunflower, the infection hyphae invade the leaf intracellular spaces and spread toward the midrib and the petiole. Xylem elements are less affected, but the phloem and parenchyma tissues are successively disintegrated on the leaf – petiole – stem route of plant infection. It might be speculated that the darkening of leaf veins, which occurs prior to the appearance of steam canker, could be the result of β -glucosidase action on conjugated phenols, as has been suggested for *Fusarium oxysporum* [26].

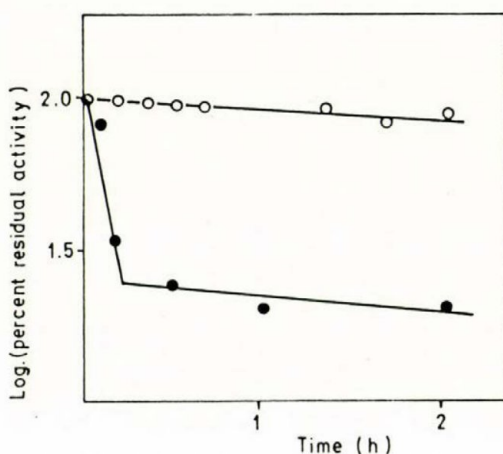


Fig. 5. Comparison of thermostability at 60 °C, pH 4.8 for intracellular (O), and extracellular (●) β -glucosidase, produced during fermentation in submerged cultivation

The above information about the capability of pathogenic fungus *P. helianthi* to produce β -glucosidase and their characterization, could contribute to a better understanding of the mechanism of pathogenic action of this fungus via CWDE and phytotoxins [27, 28] could not be excluded.

Acknowledgements. The authors wish to thank Dr. V. Leskovac, and Dr. M. Mihalčević for critical review of the paper.

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EPIFLUORESCENT MICROSCOPY OF EARTHWORMS' INTESTINAL BACTERIA*

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(Received July 30, 1993)

(Accepted February 17, 1994)

Epifluorescent microscopy was employed to compare the bacterial live counts (BC) in the gut of two earthworm species *Aporrectodea caliginosa* and *Lumbricus rubellus*, representing different ecophysiological groups. The average number of BC was $10.9 \times 10^9 \text{ g}^{-1}$ dry weight in the gut of *A. caliginosa*, 5.9×10^9 in that of *L. rubellus*, 8.1×10^9 in earthworm casts and 6.0×10^9 in the soil. The number of BC showed a great seasonal variability in all the materials studied, exhibiting maxima in spring and autumn, and a minimum in summer. The BC increased in number during the passage of food material through the gut of both *L. rubellus* and *A. caliginosa*. The difference between BC in fore-gut and hind-gut were significantly higher in *L. rubellus* (4.2×10^9 vs. 8.8×10^9) than that in *A. caliginosa* (10.3×10^9 vs. 13.4×10^9). Interspecific differences in the number of BC may result from the different chemical and microbiological composition of the material consumed by earthworms as related to different feeding habits of both species.

Most of the accumulated evidence indicate that earthworms are dependent upon a range of bacteria, actinomycetes, fungi and protozoa in their nutrition and many of these microorganisms are probably important food materials for the worms [1]. The numbers of certain types of bacteria were often found to increase within the earthworm intestine but at least some groups of microorganisms were apparently unable to survive the passage through the gut in viable forms [2–7].

Intestinal populations of bacteria and the dynamics of their densities have been studied mainly by plate count techniques [3, 4, 8]. However, it is known that only a certain fraction of gut bacteria can grow on agar plates [9]. Krištúfek et al. [10] employed a particular direct-count method using fluorescent dye and epifluorescent microscope in their study of changes in bacterial densities during passage through the

*Based on presentation at the "Second International Colloquium on Microbiology in Poikilotherms" organized by the Hungarian Academy of Sciences and L. Eötvös University, Budapest, June 15–17, 1992

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guts of different earthworm species. They demonstrated a close correlation between the intensities of microbial proliferation and the quality of consumed food in the gut contents of litter-feeding and humus-feeding earthworms.

The aim of the studies presented below was to obtain more information on the population dynamics of the earthworms' gut microbial communities. Epifluorescent microscopy was used to study bacterial counts (BC) in the digestive canals of specimens of two different earthworm species during a two-years long field investigation course and to compare the obtained data with BC of surface deposited earthworm casts and those of the surrounding soil matter.

This method can provide deep insight into the population dynamics of complex bacterial communities, in spite of the fact that it does not distinguish the bacterial cells from the hyphal bits of fragmenting actinomycetes which were found also in earthworms' gut milieus [11].

Materials and methods

The study site was an allochthonous sparse wood (*Populus nigra* L. and *Alnus glutinosa* (L.) Gaertn.) in the Stromovka-park at České Budějovice, South Bohemia, Czech Republic. The sward vegetation was dominated by *Urtica dioica* L., *Filipendula ulmaria* (L.) Max and *Carex brizoides* L. The soil type was gleyic cambisol, the soil pH (H₂O) 4.4. The climate of this area is moderately warm, average annual temperature and rainfall are 8.7 °C and 530 mm, respectively. The site was chosen because its soil, rich in leaf litter and moisture content (38%), supports a large earthworm populations almost during the whole year.

Earthworm specimens were collected monthly from March 1990 to November 1991, and at the same time surface deposited casts (unknown age and origin) furthermore soil samples from the upper 10 cm deep layer were also taken. The earthworms obtained by digging and hand sorting were transported into the laboratory and, after they were identified, representatives of two ecological groups, *Lumbricus rubellus* Hoffmeister, 1843 (epigeic) and *Aporrectodea caliginosa* Savigny, 1826 (endogeic), were used for gut content analyses. Two to three individuals of both species were surface disinfected, dissected, and their intestines were aseptically prepared. Samples of gut contents were then taken from three sections of the intestine (fore-gut, mid-gut and hind-gut). All the samples of soil, earthworm casts and gut contents were weighed, suspended in sterile water, ultrasonically homogenized (50 kHz, 4 min), and serially diluted.

For estimating bacterial counts a modified acridine orange (AO) staining method [12] was used by epifluorescence microscopy on Synpor filters. The total counts were gained with AO staining procedure (2 ml aliquots of appropriate dilutions were mixed with 2 ml of 5 mM NaHCO₃ buffer and 4 ml of 0.01% AO-solution for 30 s) and concentrated on nitrocellulose membrane filters (Synpor 8, pore size 0.23 µm, prestained with Wofalane black dye: Duha II). The green fluorescing bacteria were counted in 50 randomly chosen fields (diameter of each was 40 µm). The mean value for 1 g dry sample was calculated; dry weights of samples studied were obtained gravimetrically (105 °C). The differences among soil samples, casts, and gut contents of earthworms were calculated by means of the analysis of variance (one-way ANOVA, $P < 0.05$).

The decomposition of cellulose was detected using nylon mesh bags (10×10 mm mesh size). At the study site, cellulose sheets (five replicates) were buried in the soil at 5 cm depth and exposed for 5 weeks. Retrieved cellulose was dried to constant weight at 105 °C and ashed at 500 °C over night. All results were calculated on the basis of oven-dry weight of material, corrected for ash content.

Results and discussion

Mean values of bacterial counts (BC) and their 95% confidence limits in soil samples, casts and gut contents of earthworms are given in Fig. 1, summarizing data obtained from 17 samplings. Average BC were 10.9×10^9 g⁻¹ dry weight in the gut of *A. caliginosa*, 5.9×10^9 in that of *L. rubellus*, 8.1×10^9 in earthworm casts and 6.0×10^9 in the soil. The BC increased during the passage of food material through the gut of both earthworm species. However, the difference between BC in fore-gut and hind-gut was significantly higher in *L. rubellus* (4.2×10^9 vs. 8.8×10^9) than in *A. caliginosa* (10.3×10^9 vs. 13.4×10^9).

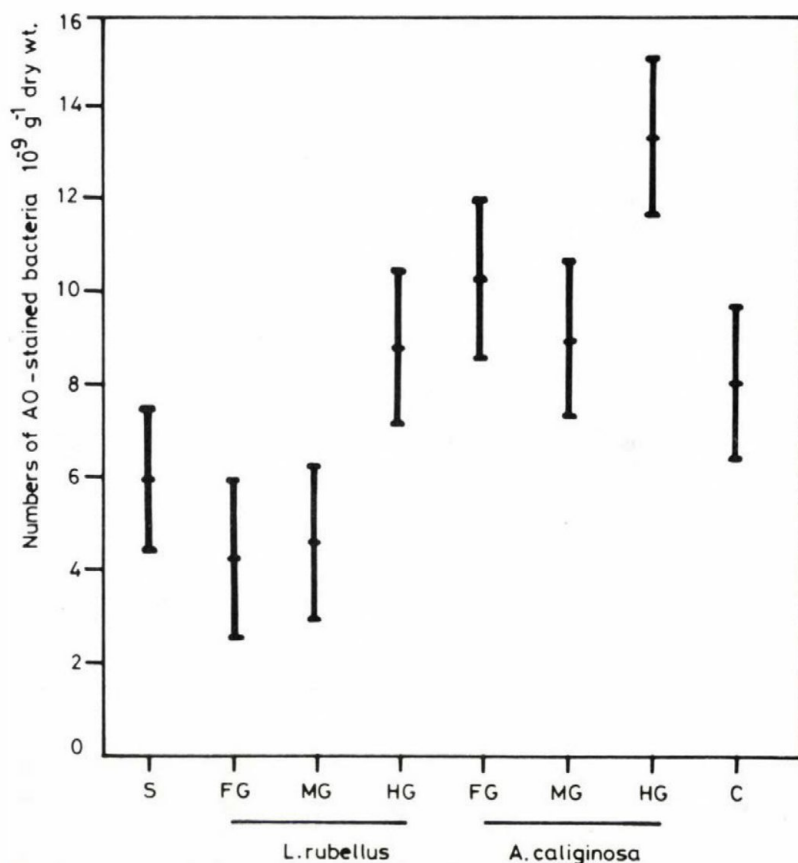


Fig. 1. 95% confidence intervals for means of the numbers of AO-stained bacteria in the soil (S), casts (C) and fore- (FG), mid- (MG) and hind-gut (HG) of *L. rubellus* and *A. caliginosa*. Summarized data from 17 samplings (1990–1991)

The seasonal fluctuations of BC were similar in both the soil matter and on the soil surface deposited earthworm casts (Fig. 2). However, the pattern of these fluctuations differed considerably during the different years. The BC followed the same general pattern as soil moisture and/or the sum of precipitation during 7 days before sampling. Two peaks of BC were observed within each year coinciding well with increased rainfall, the first occurring in spring (May 1990, July 1991) and the second one in autumn (September 1990, 1991). Soil temperature reached its maxima of 15 °C and 14 °C in July 1990 and August 1991 respectively, and it seemed to have had no direct effect on the BC fluctuation in the soil and earthworm casts.

The BC in the gut content of earthworms fluctuated according to the same pattern as the moisture or BC in the soil did, having two peaks each year. The BC fluctuations were similar in all three parts of earthworm intestines. During the second year, the fluctuations were much more pronounced than in the first one, the phenomenon being found for both *A. caliginosa* and *L. rubellus* (Fig. 2).

The decomposition of cellulose in the soil showed a periodicity with peaks in June 1990 and August 1991 (approximately 40% of the exposed weight were decomposed) and with another small peak in the autumn 1990, which was not observed in 1991, probably due to the low precipitation (Fig. 2).

Mean bacterial counts in the fore-gut of *L. rubellus* were about 33% less than those of the soil. The possible explanation could be that *L. rubellus* mainly feeds on relatively fresh plant material [13] characterized by low microbial biomass [14]. On the contrary, *A. caliginosa* is known to feed on well decomposed detritus (organic matter being ultimately mixed with anorganic soil) colonized with high numbers of microorganisms [15].

Bacterial counts in worm guts increased greatly in specimens of both species. Within the mid-gut of *L. rubellus*, the conditions seem to be more suitable for bacterial growth than those in *A. caliginosa*, where no significant increase of BC was found. The enumeration results for *L. rubellus* and *A. caliginosa* demonstrate significant increase of BC from mid-gut to hind-gut, about 100% and 50%, respectively.

Three phenomena could possibly contribute to the higher increase observed in BC over the gut content of *L. rubellus* than that of *A. caliginosa*: (1) *L. rubellus* feeds on little decomposed plant remains which are rich in organic substances. (2) Pearce [15] found that ingested material passes through the gut of *A. caliginosa* more quickly than through that of *L. rubellus*. Then, it can be supposed that the time for which the food remains in the intestine influences the proliferation of microorganisms. (3) The decrease of BC was found in the mid-gut of *A. caliginosa*. There is some evidence that soil bacteria may also be a source of food for earthworms. For instance, the numbers of *Bacillus cereus* var. *mycoides* and *Serratia marcescens* were decreasing during the passage through the earthworm gut [4], and *Escherichia coli* was killed [5].

Mean BC of the casts were about 30% higher than those in the soil. However, the origin and age of casts collected from the soil surface remain unknown.

We found close relationship between numbers of AO-stained bacteria and precipitation during the 7 days before sampling (Fig. 2). Temperature did not influence the BC, nevertheless, it had a major importance in governing the activity of soil bacteria and earthworms as was proved for the decomposition of cellulose. In contrast to BC, the highest decomposition was observed in the summer of both years.

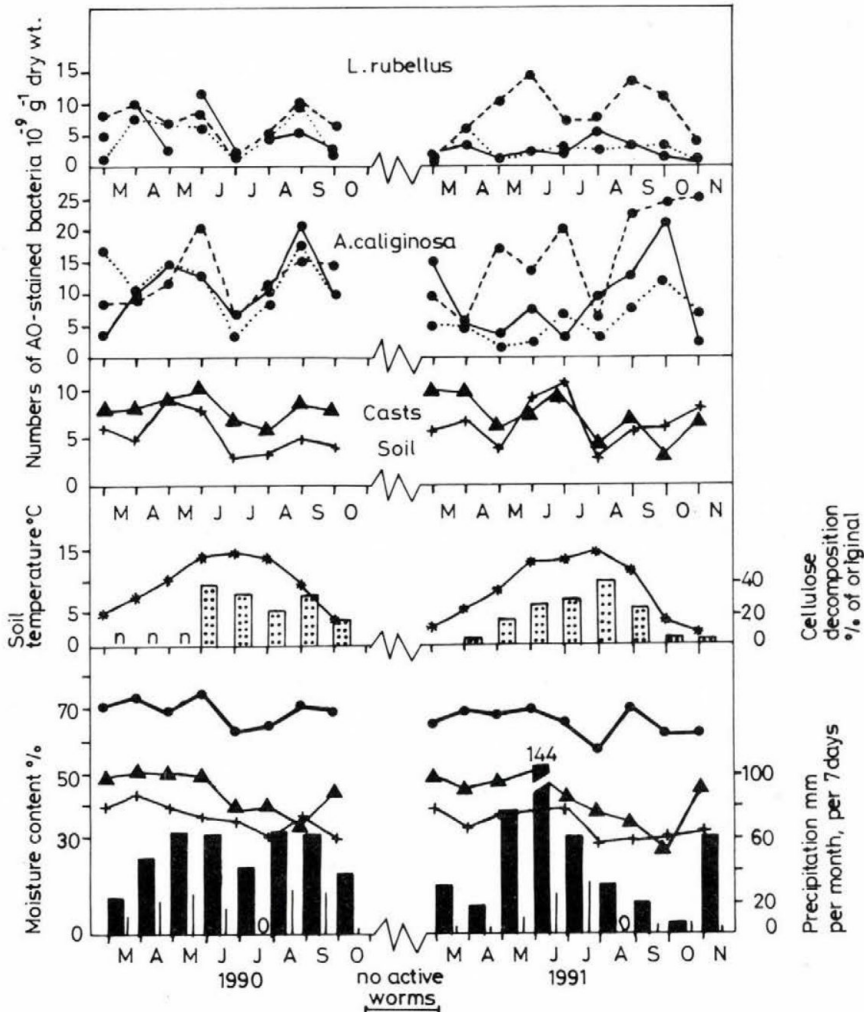


Fig. 2. Fluctuations in bacterial counts (AO-stained bacteria) in the soil, —x—; earthworm casts, —▲—; and in gut sections (fore-gut —●—, mid-gut —●—, hind-gut —●—) of *L. rubellus* and *A. caliginosa*. The average SE was 11% for AO-stained bacteria. Precipitation per month, ■, and accumulated precipitation during the 7 days before sampling, ▨, is also shown. Lack of precipitation is indicated with zero. Moisture content is shown for the soil, x, casts, ▲, and gut of earthworms, ●. The temperature of the soil, —x—, was measured in the morning. Rate of cellulose decomposition is expressed as % of original, ▨, n — not tested

Křišťůfek et al. [10] observed differences in microbial densities of different gut sections in *L. rubellus* and *A. caliginosa* (found in garden soil) as the result of the different chemical composition of the consumed food materials. Our findings were

confirmed again during the two-years field studies presented here. We demonstrated that earthworms of different ecological strategies differ not only in the quantity of the bacteria consumed with food but also in the growth rate of their gut microorganisms during the passage of the gut contents through their digestive canal.

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BACTERIA ADHERENT TO THE HINDGUT OF TERRESTRIAL ISOPODS*

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(Received July 30, 1993)

(Accepted December 14, 1993)

The gut microflora of three terrestrial isopod species was investigated by means of scanning electron microscope. The gut cuticle of intermoult specimens of *Ligidium hypnorum* and *Porcellio scaber* was densely colonized with bacteria. Some moulting specimens of the same species were observed with the guts free of bacteria. In all investigated specimens of *Hyloniscus riparius* the guts were completely free of microflora. In this case our finding could not be interpreted in relation to the moulting stage of the animal.

The intestinal tract of terrestrial isopods is one of the most interesting internal animal habitats of microorganisms. Interaction between host organism and bacteria is a well-studied topic, but still not completely understood. The possible existence of symbionts in the intestinal tract of isopod crustaceans was investigated thoroughly [1], however specialized symbionts were found only in the fish parasite *Gnathia calva* [2].

Numerous workers investigated the presence and activity of fungi and bacteria in the food, hindgut and faeces of terrestrial isopods [1, 3–11]. Boyle and Mitchell [12] reported that the gut of *Oniscus asellus* was totally free of microbial flora. Microorganisms associated with the microvillous border have been found in the hepatopancreas of terrestrial isopods [10, 13]. The selectivity of the intestinal tract for bacterial growth was documented in isopod species [7, 11]. Reyes and Tiedje [1] suggested that the isopod gut microorganisms are commensals, the gut being a favourable habitat for their growth, or that there is mutualism between the two organisms. The role of bacterial activity in isopod feeding strategy has been widely discussed. Bacteria make the consumed nutrients more readily available by decomposition [14] and by supplementing food with essential nutrients such as amino and fatty acids, vitamins and trace elements [5, 11]. Isopods are unable to degrade cellulose, so it is taught they can utilise the byproducts of activities at indigenous cellulolytic microorganisms and their cell materials [15]. The importance of bacteria in nutrition of isopods was documented, but it was difficult to separate and distinguish the

*Based on presentation at the "Second International Colloquium on Microbiology in Poikilotherms" organized by the Hungarian Academy of Sciences and L. Eötvös University, Budapest, June 15–17, 1992

microorganisms consumed with food from the members of the true indigenous microflora.

The interaction and communication between the indigenous microflora and the gut of terrestrial isopods is unique among other arthropods, because in contrast to others, the alimentary canal of terrestrial isopods is entirely lined by a cuticle. The hindgut of isopods is a digestive pathway and a specialized organ for osmoregulation [16]. In this region digestive glands are of outstanding importance. It was suggested that the gut also plays part in the absorption of nutrients [15]. The absorptive function of the gut was demonstrated only in the anterior part of the gut of three isopods [17]. The cuticle covering the gut epithelium is permeable only for smaller molecules.

Most of the studies of indigenous microflora were limited to the biochemical and physiological testing of bacterial isolates. Little is known about the factors regulating the population densities and their localization in gut microhabitats. In the alimentary canal the microbes can be intimately associated with the epithelial surface or even physically attached to them [18]. In the studies of microorganisms of the alimentary canal isopods have attracted little attention. In our work we quantitatively analyzed the gut microflora in specimens of three terrestrial isopod species using scanning electron microscope.

Materials and methods

Adult intermoult and moulting specimens of *Ligidium hypnorum* Cuvier 1792, *Hyloniscus riparius* Koch 1838 and *Porcellio scaber* Latreille 1804 were taken from their natural habitats near Lasko in Slovenia. For scanning electron microscopy the animals were fixed in modified Karnovsky [19] fixative and followed by OTOTO method [20]. The specimens were coated with gold and examined with a JEOL 840A electron microscope.

Results

The guts of *Ligidium hypnorum* and *Porcellio scaber* are densely colonized with bacteria. In *Ligidium hypnorum* the population of rod-shaped bacteria (Fig. 1) seems to be tightly associated with the gut cuticle and is very homogeneous.

In *Porcellio scaber* the distribution of bacteria along the gut is irregular (Fig. 2a, 2b). The anterior chamber of the gut the microorganisms occur very sparsely not attached tightly to the cuticle. In the papillate region of the gut dense population of microorganisms was observed, the members of which varied in shape and in the way of the attachment to the cuticle. Some bacteria were localized to the tip of the cuticular spines (Fig. 3), which protruded into the lumen of the gut. No microbes were observed in rectum. The gut of *Hyloniscus riparius* was free of bacteria in all investigated specimens (Fig. 4a, 4b).

Some specimens of *Ligidium hypnorum* (Fig. 5) and *Porcellio scaber* (Fig. 6) were also devoid of microorganisms in their guts. These specimens were examined a few hours after moulting. The gut of a specimen of *Porcellio scaber* was investigated after the posterior moult. The cuticle of the gut is intensively folded in premoult in contrast to the intermoult animals.

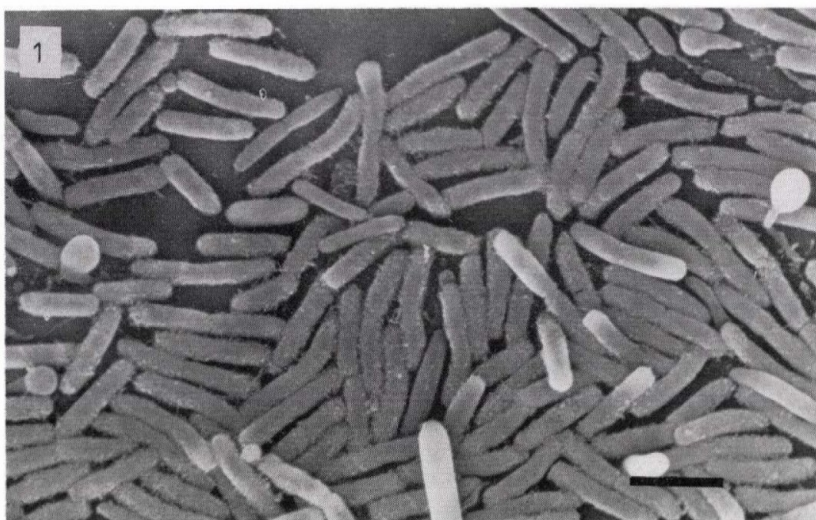


Fig. 1. *Ligidium hypnorum*. Anterior part of the gut of an intermolt animal is densely covered with rod-shaped bacteria (bar 1 µm)

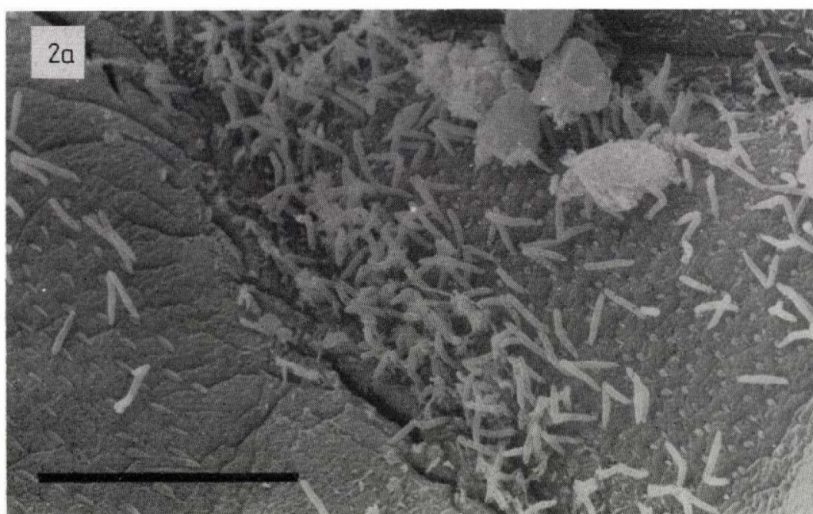


Fig. 2a. *Porcellio scaber*. Middle part of the gut of an intermolt animal is covered with bacteria (bar 10 µm)

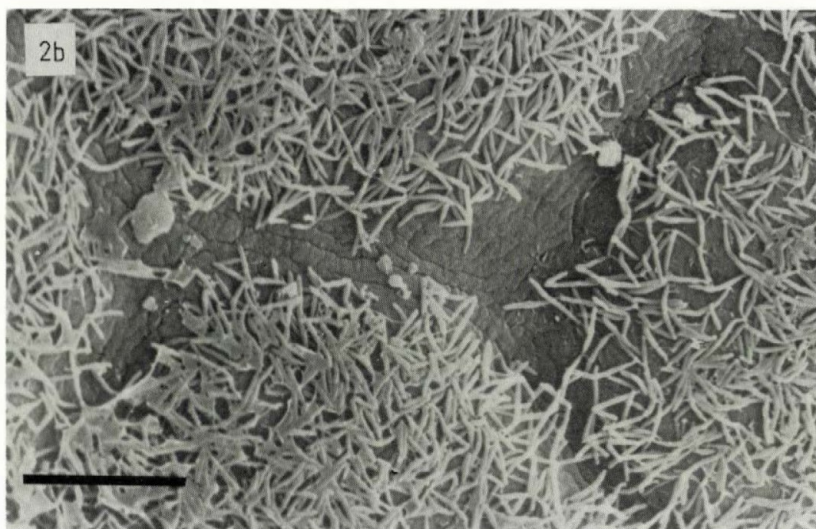


Fig. 2b. *Porcellio scaber*. Posterior part of the gut of an intermoult animal is covered with bacteria (bar 10 μm)

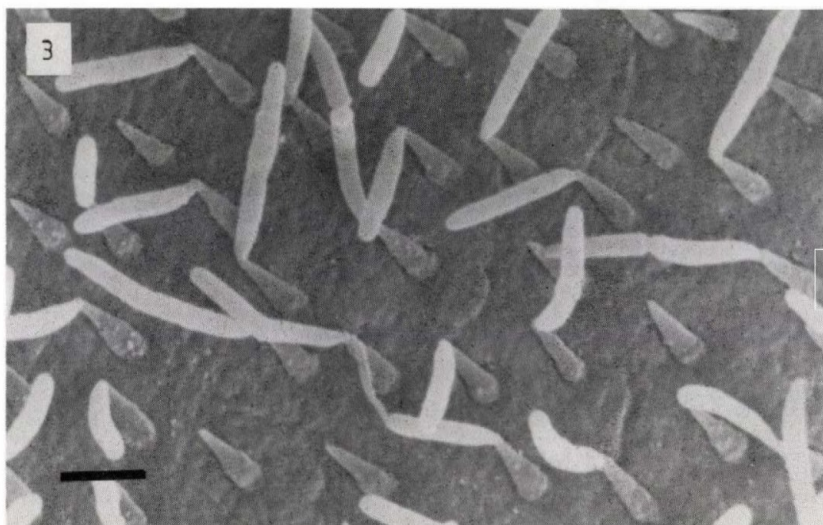
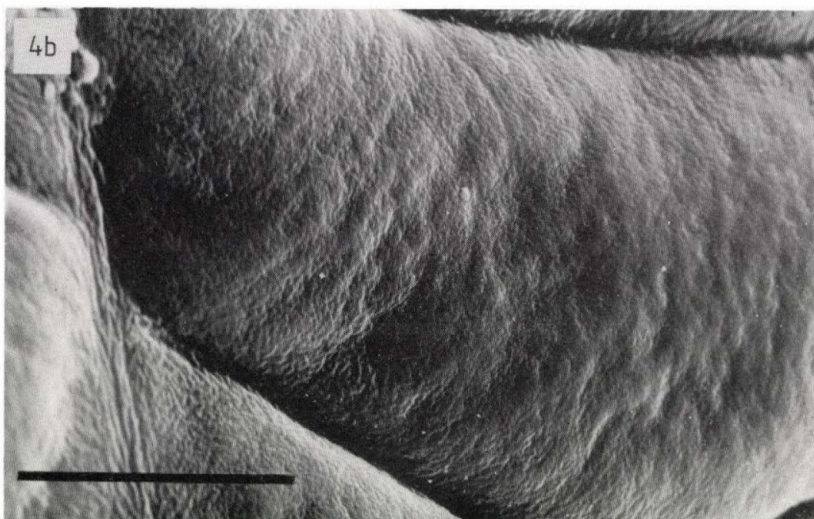


Fig. 3. *Porcellio scaber*. Some bacteria are attached to the tip of the cuticular spines (bar 1 μm)



Figs 4a, 4b. *Hyloniscus riparius*. Anterior (a) and posterior (b) part of the gut cuticle of an intermoult are completely free of bacteria (bar 10 μ m)

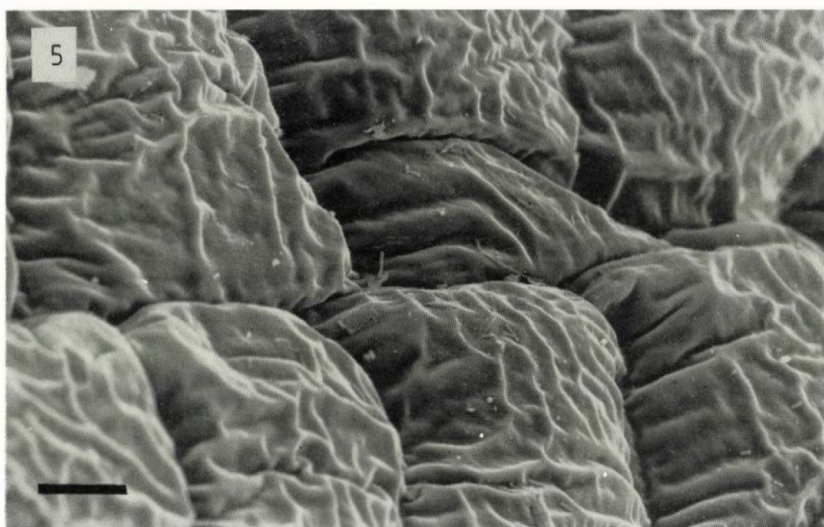


Fig. 5. *Ligidium hypnorum*. Gut epithelium some hours after moulting is covered only with a few microorganisms (bar 10 μ m)

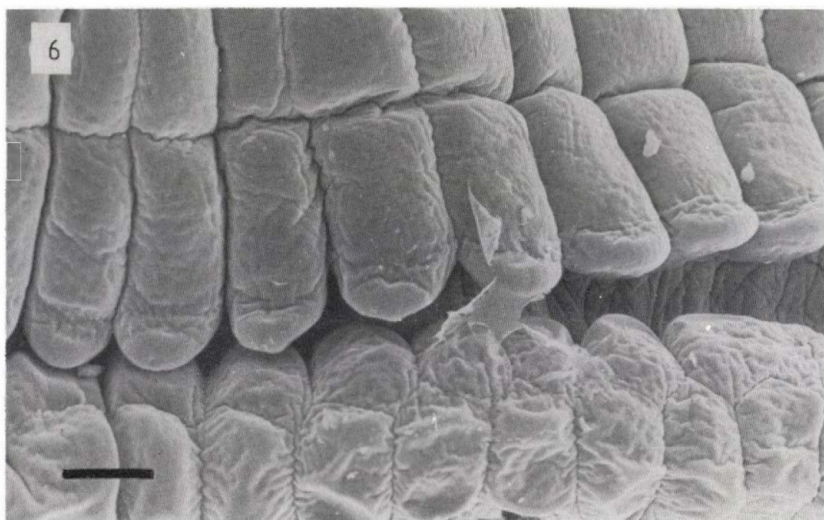


Fig. 6. *Porcellio scaber*. Middle part of the gut some hours after moulting is free of bacteria (bar 100 μ m)

Discussion

The gut of terrestrial isopods is not primarily involved in food absorption due to the cuticular barrier of the epithelial cells. The environmental conditions responsible for the existence of gut microflora in isopods differ from those in other arthropods. Therefore the benefits coming from bacterial degradation processes in the gut are limited for the host organism. However terrestrial isopods have evolved particular mechanisms for exploiting the products of bacterial degradation. One of them is coprophagy. The other is a morphological differentiation of the anterior part of the gut. Typhlosolis with paired channels enables the animal to recycle and retain fluids within the digestive system. Liquids and fine food particles, together with products of bacterial degradation, could be directed from the papillate region of the gut via typhlosole channels back to the stomach and to digestive glands. Typhlosole channels are not permanent structures in primitive terrestrial isopods such as *Ligidium hypnorum*.

Griffiths and Wood [8] suggested that bacteria ingested with food are more important than bacteria which adhere to the gut cuticle of the host organism and are distributed sparsely and irregularly along the gut. These authors have explained that the adherent bacteria simply exploit the nutrients released during digestion. However the exact function of adherent microorganisms needs to be investigated further, as well as the nature of the ability of these microbes to attach to the epithelial surfaces. It seems to be obvious from our SEM studies that there must exist a particular mechanism by which the microbe can remain in close association with the gut cuticle or even adherent to the cuticular spines. Beside the mechanism that enables the association between the bacteria and the gut epithelium is also unknown. One further question remains to be elucidated: are cuticular spines only the attachment sites for microbes or do the two organisms communicate directly?

Beside the guts, which were densely colonized with microorganisms, we analysed some guts of moulting animals which were completely free of bacteria. The absence of microorganisms in isopod gut was reported also by Boyle and Mitchell [12]. Our studies directed us to speculate that the absence of bacteria may be related to the stage of the moult cycle of the individual animal. This could mean that the animal must have evolved a mechanism providing a temporal sterility to avoid the harmful effects of microbes on the newly formed cuticle.

The absence of the gut microflora in *Hyloniscus riparius* cannot be explained on the basis of observations of moulting animals, because bacteria were never found. It seems likely that these isopods do not possess bacteria attached to the gut cuticle. The fact needs to be studied further.

Acknowledgements. The author is grateful Dr. Jasna Strus for critical comments and Dr. Kazimir Draslar for his help in SEM.

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HYBRID PROCESS FOR ETHANOL PRODUCTION FROM RICE STRAW

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(Received September 6, 1993)

(Accepted January 3, 1994)

A hybrid process for the fermentation of rice straw hydrolysates into ethanol was designed to simultaneously utilize cellulose and hemicellulose fractions of the agro-residue. The process involved dilute acid hydrolysis (for obtaining C-5 sugars) followed by enzymatic saccharification of cellulose enriched fraction with crude cellulase produced by mixed cultures of *Trichoderma reesei* Rut C-30 and *Aspergillus ochraceus* IMI 317911. The fermentation medium containing acid and enzymatic hydrolysate mixture of pentoses and hexoses monomers was fermented with yeast coculture of *Saccharomyces cerevisiae* and *Pachysolen tannophilus* resulting in 295.0 ml ethanol/kg of rice straw. The hybrid process resulted in an efficient utilization of both cellulosic and hemicellulosic components of the rice straw for ethanol production.

India has abundant lignocellulosic agrowastes amounting to 300 MT annually, of which rice straw contributes 90 MT [1]. Rice straw is rich carbohydrate base that can be bioprocessed into ethanol, an energy-rich liquid fuel. However, the bioconversion process involves concerted approach involving four steps namely, pretreatment, enzyme production, saccharification and ethanol fermentation. Each of these steps requires considerable research and development efforts to make the process cost competitive with the existing molasses and starch based alcohol production.

Although lignocellulose is cheap and abundant, further steps are required to achieve fermentable sugars. These steps are pretreatment, enzyme production and saccharification which contributes 52% to the overall process cost, of which cellulase

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production cost alone accounts for 40% of the total cost involved [2, 3]. Therefore, enzyme based technology can be made cost competitive only if the cellulase production cost is cut down significantly. To date, research efforts directed towards bioconversion of lignocellulosics to ethanol have not attained commercial status and even the technically most feasible process available at pilot scale (Arkansas, Natick), utilises only the cellulosic fraction (30-40%) of the biomass as the sugar base [2]. However, for making such a biomass based ethanol process economically viable, simultaneous conversion of pentoses alongwith hexoses into ethanol is required [4]. The hydrolysis of hemicellulose to pentoses requires low activation energy and thus can be easily removed from lignocellulosic complex using mild acids. The monomeric sugars obtained as pentoses in the hydrolysate can be fermented to ethanol by *P. tannophilus*, *Candida shehatae*, *Pichia stipitis* etc. [5]. Since, the hydrolysate obtained after partial/enzymatic hydrolysis contains mixture of sugar monomers. Therefore, the present study has been attempted to utilize both pentoses and hexoses fractions for bioconversion to ethanol by employing a hybrid process.

Materials and methods

Substrate. The rice straw was ball-milled to a uniform particle size (2 mm) and was analysed for its cellulose, hemicellulose and lignin content on dry matter basis and was subsequently used as substrate in the present studies.

Dilute acid hydrolysis. Fifty grams of substrate was soaked in an Erlenmeyer flask (1 litre capacity) containing 650 ml of dilute sulphuric acid (concentration 0.3, 0.4 or 0.5%) solution. The substrate was steam treated in an autoclave at 20 lb/in² at 121 °C for different reaction time (15, 30, 45 or 60 min) after which the steam-release valve was opened for immediate depressurization. The resultant acid hydrolysates were filtered and neutralized using lime. The hydrolysates and the resultant residues were analysed for their chemical composition.

Cellulase production. The fungal cultures of *T. reesei* Rut C-30 and *A. ochraceus* IMI 317911 were used for cellulase production as described previously [6].

Enzymatic saccharification. The crude enzyme aliquots were used for the hydrolysis of wet-cellulose enriched fraction obtained after acid hydrolysis. The hydrolysis was performed in a 250 ml Erlenmeyer flask (working volume 100 ml) containing 6.67 g substrate (dry wt.), 50.0 ml crude enzyme and 47.0 ml citrate buffer (0.5 M; pH 4.8) at 50 °C for 72 h. After the incubation period, contents of the flasks were centrifuged at 3000 g for 10 min and the supernatant was analyzed for reducing sugars.

Ethanol fermentation. (a) *Acid hydrolysate medium.* The acid hydrolysate was neutralized with lime followed by filtration. The resultant hydrolysate was subjected to solvent extraction using diethyl ether as solvent for removal of organic acids, lignin degradation products, tannins and furfurals. This hydrolysate was referred as treated hydrolysate. (b) *Mixed hydrolysate.* This preparation contained equal amounts of enzymatic and treated acid hydrolysate.

Medium ingredients. The medium "a" and "b" were supplemented with (g/l): Yeast extract, 10.0; KH₂PO₄, 1.2; MgSO₄, 0.5; CaCl₂, 3.0 and trace mineral solution (10.0 ml) containing (g/l) ZnSO₄, 0.4; FeCl₃, 0.4; CaSO₄, 0.25; H₃BO₃, 0.06 and conc. HCl, 10.0 ml.

Microorganism and culture media. The yeast culture *P. tannophilus* NRRL 4160 Y was kindly provided by Dr. Kurtzmann, NRRL, Illinois (USA) and *S. cerevisiae* was obtained from the Department of Microbiology, Punjab Agricultural University, Ludhiana (India). These cultures were maintained on GYE medium at 4 °C. The inoculum was prepared in 250 ml flasks containing 100 ml of GYE broth except glucose was replaced with D-xylose for the growth of *P. tannophilus*. The flasks were incubated at 30 °C for 24 h under shaking conditions.

Batch fermentation. The fermentation was carried out in a bench top sterilizable fermentor (Model KLF 2000, Bioengineering) with working volume of 2 litres. The fermentor containing 800 ml of medium "a" and "b" was co-inoculated with 40 ml of *S. cerevisiae* + *P. tannophilus* inoculum taken in 1:1 ratio with direct microscopic count of 2×10^8 cells/ml. The fermentor was operated at 28 °C and contents were stirred at 100 rpm. The pH of the medium was controlled at 5.0 by addition of an appropriate amount alkali (2.0 M) whenever required using a peristaltic pump (Micro tube pump MP-3). The dissolved O₂ level was regularly monitored and maintained at 2 ppm during the course of fermentation.

Analytical methods. The cellulose [7], hemicellulose and lignin [8] were analysed as previously reported. Xylose determined by a modified orcinol method [9]. The total reducing sugars were estimate by Miller's method [10]. The furfural(s) concentration in the acid hydrolysate was determined after developing red colour with aniline and absorbance was read at 600 nm [11]. The ethanol contents were determined by gas liquid chromatography using a Chromosorb-102 column [12].

Results

Substrate. The rice straw was analysed for cellulose (39.0%), hemicellulose (30%) and lignin (9.6%) content. The physical treatment (ball-milling) of rice straw resulted in the increase in bulk density from 43.85 to 147.14 kg/m³.

(i) **Hemicellulose fractionation.** The rice straw was hydrolysed with different sulphuric acid concentrations (solid:liquid ratio of 1:13) for varying reaction time and was autoclaved under steaming pressure (20 lb/in²). The results in Table I showed that an increase in acid concentration and reaction time had a positive effect on the yield of total reducing sugar and furfurals. A maximum reducing sugar yield (0.576 g/g) equivalent to 83% carbohydrate recovery was achieved at 0.5% (v/v) acid concentration after 60 min of reaction time. However, it was accompanied with high amount of furfurals (0.69%, v/v) in the hydrolysate. In contrast, the dilute acid treatment with H₂SO₄ (0.4%, v/v) for 30 min brought about an almost complete solubilization of hemicellulose (95.0%) and cellulose (50.0%). The resultant acid hydrolysate containing reducing sugars (0.487 g/g; out of which xylose constituted 0.287 g/g) without any detectable furfurals, was used for subsequent alcoholic fermentation. The rice straw residue obtained following dilute acid hydrolysis contained 58.2% cellulose, was enzymatically hydrolysed with a crude cellulase extract obtained from a mixed cultivation of *A. ochraceus* and *T. reesei*-Rut C-30.

(ii) **Cellulase production and saccharification.** A balanced cellulase enzyme complex (1.89 IU/ml FPA and 1.63 IU/ml cellobiase) was produced by fermenting basal medium containing rice straw (6.0%; w/v as carbon source) involving a mixed culture of *T. reesei* Rut C-30 and a hypercellobiase producing *A. ochraceus* strain [6]. This crude enzyme complex was used for saccharification of cellulose enriched residue of acid treated rice straw. The saccharification was carried out at an initial substrate concentration of 6.67% (w/v) using an enzyme dosage of 25 IU of Filter Paper Activity and 22 IU of cellobiase/g of the cellulose fraction present in the acid treated rice straw. The enzymatic hydrolysate thus obtained yielded additional reducing sugars (0.165 g/g) for fermentation (Fig. 1).

Table I
Optimisation of dilute acid hydrolysis

H ₂ SO ₄ conc. (%, v/v)	Reaction time (min)	Total sugars (mg/g)	Pentoses (mg/g)	Furfural (%, v/v)
0.1	15	231	187	—
	30	287	217	—
	45	350	235	—
	60	403	252	—
0.2	15	307	193	—
	30	354	237	—
	45	407	255	—
	60	430	252	—
0.3	15	308	265	—
	30	433	285	—
	45	457	293	—
	60	480	280	0.33
0.4	15	367	272	—
	30	487	287	—
	45	512	282	0.33
	60	535	272	0.56
0.5	15	463	286	0.26
	30	493	283	0.59
	45	560	280	0.66
	60	576	266	0.69

Reaction conditions: working volume, 650 ml (solid:liquid = 1:13); temperature, 121 °C; 20 lb/in²

(iii) *Ethanol fermentation*. The acid hydrolysate containing a mixture of reducing sugars (48.7 g/l) was fermented to ethanol using coculture of *P. tannophilus* and *S. cerevisiae*. However, no alcohol was produced even after 3 days of incubation at 30 °C. Therefore, the hydrolysate was subjected to a solvent extraction treatment to improve fermentability of acid hydrolysate. The treated hydrolysate containing 45.0 g/l of total sugars was fermented to ethanol under optimised conditions, with a coculture of *P. tannophilus* and *S. cerevisiae* which yielded an ethanol content (16.0 g/l) amounting to 203.0 ml/kg rice straw.

(iv) *Hybrid process*. In the subsequent experiment the treated acid hydrolysate was pooled with the enzymatic hydrolysate. The pooled hydrolysate containing reducing sugars (36.0 g/l) was fermented to ethanol employing yeast coculture. After 72 h of fermentation, the ethanol content (14.5 g/l) amounted to 295.0 ml ethanol/kg of rice straw.

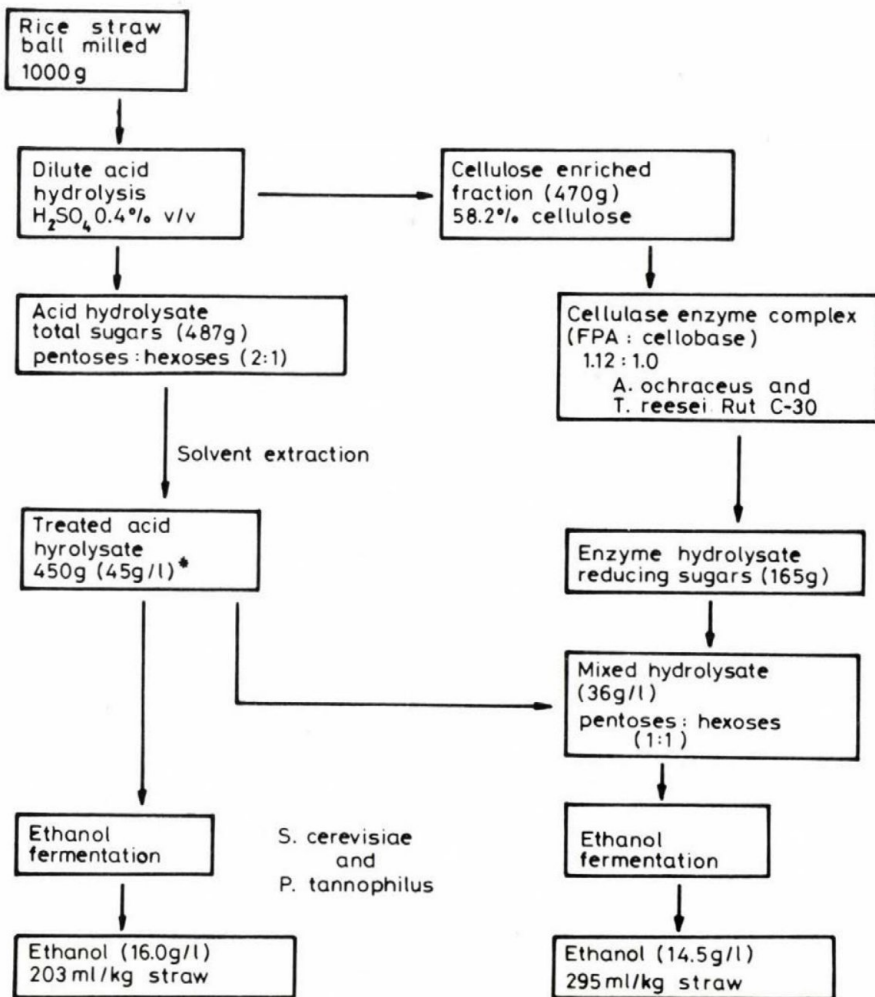


Fig. 1. Hybrid process for ethanol fermentation. Rice straw (milled) was subjected to dilute acid hydrolysis in Step I. The cellulose enriched fraction was enzymatically hydrolysed using a balanced cellulase enzyme complex. The mixture of acid and enzyme hydrolysate was pooled and fermented to ethanol using a co-culture of *S. cerevisiae* + *P. tannophilus*. The method involving use of both pentoses and hexoses was efficient as compared to the pre-existing process(es) which utilize(s) cellulose fraction only (2).

*Total reducing sugars

Discussion

The suitability of rice straw as a good carbon source is well recognised [13]. However, physical treatment of the lignocellulosics is required to make it susceptible to an acid and enzymatic hydrolysis to yield fermentable sugars. The physical treatment is known to decrease the cellulose crystallinity besides exposing more surface area which effect(s) the removal of hemicellulosic fraction as pentoses; and partially the cellulosic fraction as hexoses [14]. The partial/dilute acid hydrolysis when carried out for a longer period results in appreciable amounts of furfurals [12] which even at low concentration (0.2%; v/v) are toxic to the yeast metabolism affecting its fermentation efficiency [15]. In addition, other toxic degradation products such as acetic acid, formic acid, tannins, phenolic compounds etc. are also present in the hydrolysate that inhibit the fermentation process [16]. The method of solvent extraction (diethyl ether) for treating acid hydrolysates was fairly effective for the removal of toxic compounds from acid hydrolysates which could be efficiently fermented to 203.0 ml ethanol/kg of rice straw. The ethanol content was almost equal to the previously reported value (190–220 ml/kg) from existing technology based on using cellulose fraction of the residue only [2].

The hybrid process described in the present study employed a mixture of treated acid hydrolysate and enzymatic hydrolysate. The enzymatic hydrolysis of the cellulose enriched fraction of acid treated straw with a balanced cellulase enzyme complex derived by co-culture of *T. reesei* + *A. ochraceus* resulted in an efficient saccharification leading to availability of more fermentable sugars in the form of hexoses. We have previously reported that a fermentation medium containing mixture of D-xylose and D-glucose in equal amounts can be effectively fermented to ethanol by yeast coculture of *S. cerevisiae* and *P. tannophilus* [17]. Therefore, the fermentation of mixed hydrolysate containing hexose:pentose in the ratio of 1:1 was fermented using yeast coculture that resulted an ethanol yield of 295 ml/kg of rice straw which is considerably higher than previously reported [2, 12]. Therefore, hybrid process using both C₅ and C₆ sugars as advocated by Felber [4] and Klyosov [2] has been found to be suitable for developing an economically feasible lignocellulose based bioconversion process.

Acknowledgement. The financial support extended by The School of Energy Studies for Agriculture, P.A.U., Ludhiana (India) in the form of ICAR/UNDP sponsored Senior Research Fellowship for pursuing this study is duly acknowledged.

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EFFECT OF SOME ENVIRONMENTAL FACTORS ON *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS*

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(Received September 9, 1993)

(Accepted January 3, 1994)

Effects of different abiotic factors (acidity, salinity, nitrate and temperature) on growth rate of root-nodule bacteria (*Rhizobium* and *Bradyrhizobium*) strains were investigated in vitro. Strains isolated from *Vicia faba* L., *Coronilla varia* L. and *Lupinus albus* L. exhibited a large variation in tolerance of the above-mentioned factors. These bacteria should be screened under stimulated conditions for enhanced survival before selection to be used for commercial inoculant production. Linear correlation matrix data were useful to find the appropriate concentrations for the selection of the tolerant strains.

One of the most important aims of soil biotechnology is to reduce the agricultural environmental pollution using microorganisms instead of chemicals for soil fertility. The benefits from inoculation mostly depend on the survival of introduced rhizobia in the soil [1]. They must survive long enough after sowing to nodulate the host plant and, preferably persist between cropping seasons. Ecological factors such as soil nitrogen-content, temperature [2], acidity [3–5] and salinity [6, 7] are the main factors that strongly influence the efficiency of root-nodule bacteria. The limiting levels of these factors, however show a wide variation when considering different host plants and microsymbionts. Lie [8] mentioned that leguminous plants, with the exception of the cowpea and lupin groups, failed to nodulate in acidic soils. Aarons and Graham [9] isolated *Rhizobium leguminosarum* bv. *phaseoli* strains with increased pH-tolerance. Recently, other *Rhizobium* strains with higher pH-tolerance have been identified for most rhizobial species [10–15] Wolf et al. [16] reported that the tropical

*This paper was written in honour of the memory of Professor Zoltán Alföldy (1902–1992) director emeritus of the Institute of Microbiology Semmelweis University Medical School in Budapest (Hungary)

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strains of bean rhizobia had a high tolerance towards low pH. Howieson et al. [12] found that strains of *R. meliloti* isolated on low pH media were in general more acid-tolerant than related isolates obtained from media with higher pH. Chen et al. [17] identified an acid-tolerant strain of *R. leguminosarum* bv. *trifolii* with enhanced capacity for nitrogen fixation. Batzil et al. [18] mentioned that most *Rhizobium* strains preferred neutral rather than acidic pH. Hodgson et al. [1] reported that fast-growing rhizobial species can better tolerate soil salinity.

High and low temperatures can affect the performance of soil microorganisms [2] and the necessity of selecting bacteria for pH tolerance [5] has also been demonstrated. Regarding N-fertilization, Hardarson et al. [19] found that bean and soybean are very sensitive while horse bean can tolerate up to 400 kg N per ha fertilizer too [20]. Therefore, a number of abiotic factors can affect in many ways both microsymbionts and host plants. The objective of our investigation was to evaluate in vitro the different sensitivity to ecological factors of some *Rhizobium* and *Bradyrhizobium* strains isolated from various host plants and soil types.

Materials and methods

Strains. Five strains of *R. leguminosarum* (three bv. *viciae*, one bv. *phaseoli* and one bv. *trifolii*) five strains of *R. loti* and five strains of *Bradyrhizobium* sp. (*Lupinus*) were used (Table I). Rhizobial and Bradyrhizobial growth characteristics were observed in Yeast Extract Mannitol (YEM) broth medium [21].

Salinity and nitrate tolerance were examined by growing the strains in YEM broth, containing 0, 100, 200, 300 and 400 mM NaCl and 0, 100, 200 and 300 mM KNO₃. Cell density of the suspensions was followed measuring the absorbance at 550 nm in a DR-2000 spectrophotometer after 48 h incubation in microfermentor at 28 °C. To study the acidity, Sorensen [22] phosphate buffer was used and the pH adjusted to 5.0, 5.5, 6.6 and 7.0 (as control). Growth of the strains at various temperatures was tested by incubating the strains on YEM agar plates and slants at 5, 15, 20, 28 (as control), 30 and 40 °C.

Growth kinetics was monitored in YEM broth by absorbance at 550 nm. Data on optical density are expressed as percentage in comparison with controls. Variance analysis was performed. Correlation matrix of the results was calculated for checking the effect of these factors on the investigated microsymbiont of *R. leguminosarum*. Significant difference (95% probability level) of similar means of three control replicates are labelled with letter "a".

Results

Salt tolerance. Results are shown in Table II. In most of the cases, there was no effect up to 100 mM on the *Rhizobium* and *Bradyrhizobium* strains with the exception of faba bean *Rhizobium* strain 1012, clover *Rhizobium* strain L6 133/64 and *R. loti* strain K 58, which were particularly affected by NaCl stress. On the other hand, growth of *R. leguminosarum* bv. *viciae* strain L6bab Z was stimulated even at 400 mM. Experiments are currently in progress to establish the upper limits of salinity tolerance of these strains. In the case of the three coronilla *Rhizobium* strains (Sz 3, G 1 and K 96) and two lupine *Bradyrhizobium* strains (Br 14 and Br 31) the limit, proved to be

200 mM, while the remaining strains could tolerate only 100 mM NaCl. In terms of sensitivity there were no differences at the level of 100 mM between the strains of fast- and slow-growing microsymbionts.

Table I
Experimental microsymbiont strains

Root-nodule bacteria	Lab, code and origin	Host plant
<i>R. leguminosarum</i> bv. <i>viciae</i>	HB-3841 (Libya) L6bab Z (Hungary) 1012 (UK)	<i>Vicia faba</i>
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	Bab 5/3 (Hungary)	<i>Phaseolus vulgaris</i>
<i>R. leguminosarum</i> bv. <i>trifolii</i>	L6 133/6 (Hungary)	<i>Trifolium repens</i>
<i>R. loti</i>	Sz 1 (Hungary) G 1 K 58 K 93 K 96	<i>Coronilla varia</i>
<i>Bradyrhizobium</i> sp. (<i>Lupinus</i>)	Br 1 (Hungary) Br 8 Br 14 Br 29 Br 31	<i>Lupinus albus</i>

pH tolerance. A marked difference was noted within the native population of coronilla *Rhizobium* e.g. between strains G 1 and K 96 (Table III). Among the *Bradyrhizobium* strains, there were significant differences in the most acidic media as well. All the fifteen strains tested in the defined medium grew to some extent at pH 5.0 or above, some were very affected in their growth rate. Comparing the effect of acidity among the fast- and slow-growing bacteria, the latter showed a better tolerance of the most acidic media (mean at pH 5.0 is 43.7% for *Rhizobium* strains, and 64.1% for *Bradyrhizobium* strains). On the other hand, coronilla *Rhizobium* strains were not as sensitive as *R. leguminosarum* bv. *viciae* strains. *R. leguminosarum* bv. *viciae* strain L6bab Z, *R. loti* strain K 96 and *Bradyrhizobium* sp. (*Lupinus*) strains Br 31 and Br 8 were particularly acid-tolerant.

Effect of mineral nitrogen. All tested strains, with the exception of Br 31, were affected by 300 mM KNO₃ (Table IV). At this concentration, *R. leguminosarum* strains were more sensitive than *Bradyrhizobium* sp. (*Lupinus*) and crownvetch *Rhizobium* strains (mean = 49.2, 76.9 and 78.4%, respectively). On average, the most tolerant strains proved to be among *R. loti* and *R. leguminosarum* bv. *viciae*. Strains originating from faba bean grow well at 200 mM, while among the slow-growing *Bradyrhizobium* strains Br 31 proved to be particularly tolerant to the N-content of media. A stimulating

effect was found with Lóbab Z strain at 100 mM KNO₃. At this concentration, there were no significant differences between the fast- and slow-growing strains.

Table II

*NaCl tolerance of root-nodule bacteria
(per cent of the control optical density)*

Root nodule bacterial strains	NaCl concentration (mM)			
	100	200	300	400
HB-3841	144.9	49.7	29.7	20.0
Lóbab Z	149.9	185.8	192.6	142.9
1012	60.0	40.0	10.0	7.5
Bab 5/3	87.4	47.7	34.9	6.3
Ló 133/64	18.5	14.1	13.5	11.1
Sz 3	97.2a	102.8a	74.6	10.6
G 1	102.3a	109.0a	86.1	37.6
K 58	57.0	27.0	14.3	5.1
K 93	94.9a	76.6	25.8	8.6
K 96	98.3a	91.3a	82.6	48.8
Mean for <i>Rhizobium</i>	91.0	74.4	53.5	17.3
Br 1	95.0a	54.7	40.6	25.5
Br 8	98.6a	52.2	40.0	23.1
Br 14	98.3a	91.5a	88.9	45.2
Br 29	97.0a	55.0	45.7	30.6
Br 31	125.7	109.2a	43.6	38.2
Mean for <i>Bradyrhizobium</i>	102.9	72.5	51.7	32.1
Mean (total)	95.0	73.7	52.6	25.7

a = values with letter "a" in the column are not significantly different from the control at $P < 0.05$

Effect of temperature. Table V shows the temperature effect on the different strains. According to the literature optimal values range from 20 to 30 °C. With the exception of the *Bradyrhizobium* isolates strains were not able to grow in some cases at 5 °C. As far as high temperature degree, crownvetch *Rhizobium* strains were the only ones that could not tolerate 40 °C. The slow-growing strains were the most tolerant at low, as well as at high temperatures. Among the *R. loti* strains, however, Sz 3 grew very well at 5 °C, this isolate therefore proved to be the most convenient for crownvetch inoculation before the winter period. Strains Br 8 and Br 31 are also good cold tolerant candidates.

Table III

*pH tolerance of Rhizobium and Bradyrhizobium strains
(per cent of the control – pH 7.0 – optical density)*

Root nodule bacterial strains	pH values		
	5.0	5.5	6.6
HB-3841	10.0	45.5	80.0
L6bab Z	66.0	82.1	98.3a
1012	6.8	40.7	74.5
Bab 5/3	40.0	62.2	85.0
L6 133/64	20.9	42.2	63.5
Sz 3	35.5	62.9	89.5
G 1	11.3	54.0	84.5
K 58	77.8	83.8	85.3
K 93	71.2	90.0	92.8a
K 96	96.5a	100.0a	100.0a
Mean for <i>Rhizobium</i>	43.7	63.4	85.5
Br 1	40.2	75.5	70.5
Br 8	37.4	132.0	67.4
Br 14	78.3	77.9	92.0a
Br 29	80.5	95.0	90.2
Br 31	84.3	106.2a	103.8a
Mean for <i>Bradyrhizobium</i>	64.1	97.3	84.7
Mean (total)	54.4	76.7	85.1

a = values with letter "a" in the column are not significantly different from the control at $P < 0.05$

Discussion

Tolerance of Rhizobium and Bradyrhizobium strains to various abiotic factors. Salinic soils may contain 0.8–1.3% NaCl in a depth of 180–440 mm [23]. Since salt affected soils occupy large areas of the world, utilization of salt-tolerant rhizobia could be beneficial in agricultural practice.

Acidity is a major factor affecting the growth and the distribution of native plant species. It can limit legume growth and nitrogen fixation because of its adverse effects on the root-nodule *Rhizobium* and *Bradyrhizobium* and on the symbiotic interaction. Strains of *R. meliloti* selected for acid tolerance, have been used in laboratory, greenhouse and field trials [12]. We plan to select environmentally more tolerant strains of the microsymbionts to conduct field trials. Due to the large and perhaps continuous range of rhizobial tolerance, selection should be possible among them.

Other environmental factors such as temperature and soil nitrate-content are also of primary importance. Nelson [24] has found differences in tolerance to nitrate between rhizobial isolates in the presence of NH_4NO_3 . Differences in the extent of inhibition of nodulation by nitrate have been reported [25, 26]. McNeil [27] mentioned that nitrate tolerant *R. japonicum* isolates can increase the nodule number on soybean with 30%. We also found a wide variety in response both to nitrate concentration and temperature. Survival at low and high temperatures is very important for the rhizobial inoculants, since in temperate climates, winter (-20 to -30 °C) and summer ($+30$ to $+40$ °C) temperatures can be very different.

Table IV

*Tolerance of Rhizobium and Bradyrhizobium strains to KNO_3
(per cent of the control optical density)*

Root nodule bacterial strains	KNO ₃ concentration (mM)		
	100	200	300
HB-3841	104.0a	106.1a	71.5
Lóbab Z	146.7	113.1a	56.4
1012	115.5a	120.3a	50.2
Bab 5/3	74.8	56.8	36.1
Ló 133/64	85.8	37.3	31.9
Sz 3	81.7	73.7	69.3
G 1	94.3a	92.4a	89.9
K 58	97.7a	88.5	75.4
K 93	91.3a	85.9	84.5
K 96	97.3	92.6	85.7
Mean for <i>Rhizobium</i>	98.9	78.1	68.0
Br 1	73.0	72.7	68.5
Br 8	83.4	83.3	76.1
Br 14	74.3	70.4	60.2
Br 29	95.0a	87.6	80.7
Br 31	102.3a	99.7a	99.0a
Mean for <i>Bradyrhizobium</i>	105.6	82.7	76.9
Mean (total)	102.2	79.6	72.4

a = values with letter "a" in the column are not significantly different from the control at $P < 0.05$

The first screening of these deleterious effects on microsymbionts can take place in vitro, where a collection of candidate root-nodule bacteria can be severely reduced by a single test aimed at identifying strains giving high performance under the different stress conditions.

Snehaljoshi and Nimbalkar [28] found that NaCl was more deleterious than Na_2SO_4 ; we also used various concentrations of NaCl in our trials, investigating the salt-tolerance of the strains. We found in agreement with Yadav and Vyas [29] that root-nodule bacteria may be resistant to 300 mM of salt applied to the medium. Pillai and Sen [30] were of the opinion that gum produced by *Rhizobium* strains might protect them against salt effects. Protection, however, is not a strain specific behaviour, it is observed mostly with fast-growing species. Testing the salt tolerance of ten *Rhizobium* and five *Bradyrhizobium* strains, we could notice the difference between the slow- and fast-growing strains, especially at the higher doses (300–400 mM NaCl). As regards, other factors examined in this investigation except for the nitrate tolerance, there were also differences in sensitivity between slow- and fast-growing strains of the symbiotic N_2 -fixers.

Table V

Effect of different temperatures on growth of rhizobia

Root nodule bacterial strains	Temperature (°C)				
	5	15	20	30	40
HB-3841	+	++	+++	++	++
Lóbab Z	–	++	+++	++	++
1012	–	++	+++	+++	++
Bab 5/3	+	++	+++	+++	++
Ló 133/64	–	+	+++	+++	++
Sz 3	++	++	+++	++	++
G 1	+	++	+++	++	–
K 58	–	++	+++	++	+
K 93	+	++	+++	+++	–
K 96	+	++	+++	+++	+
Br 1	–	+	+++	+++	++
Br 8	++	++	+++	+++	+++
Br 14	+	+++	+++	+++	++
Br 29	+	++	+++	++	++
Br 31	++	++	+++	+++	++

Growth on YEM agar slants: (–) nil, (+) poor, (++) good, (+++) maximum

Selecting Rhizobium and Bradyrhizobium strains for host plant inoculation. In order to select inoculants among the different root-nodule bacteria tested in this investigation, we tried to make a comparison between different results. Part of a linear correlation matrix is shown in Table VI, where we observed that the best selection of our strains may occur at the level of 100 mM NaCl. Other concentrations (200 and 300 mM NaCl) failed to indicate further differences. The same is applicable for nitrate-

tolerance, too. The effect of 200 and 300 mM KNO_3 content does not give more information for selection, since correlation data are similar. As regards acid-tolerance, only at the level of 100 mM NaCl appears an efficient selection possible.

Table VI

Correlation data of acid pH-, potassium nitrate and sodium chloride-tolerance according to the sensitivity of *R. leguminosarum* strains

	pH 5.0	pH 5.5	pH 6.6	100 mM KNO_3	200 mM KNO_3	300 mM KNO_3
100 mM NaCl	0.58199	0.62824	0.71431	0.30248	0.84460	0.68963
200 mM NaCl	0.99993	0.99891	0.98674	0.94754	0.06761	0.17626
300 mM NaCl	0.99873	0.99997	0.99231	0.93439	0.10637	0.13784
400 mM NaCl	0.99937	0.99975	0.99037	0.93957	0.09163	0.15250

The correlation significant was measured at $P < 0.05$

Among the *R. leguminosarum* bv. *viciae* strains, for inoculation of faba beans Lóbab Z proved to be especially tolerant, over a winter period the survival of strain HB-3841 could have been predicted better. Among the crownvetch *Rhizobium* strains K 93 and K 96 gave the best performance. On the other hand, in case of wide temperature fluctuation, strain Sz 3 might be more tolerant in the field trials. As the slow-growing *Bradyrhizobium* strains are concerned in terms of tolerance against the abiotic factors examined in this investigation, strains Br 31 and Br 8 were outstandingly fit especially at 5 and 40 °C.

Although care should be taken when extrapolating these in vitro results in the field, the need to find ecologically tolerant strain(s) as seed inoculants is of primary agronomical importance.

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SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF RICE STRAW INTO ETHANOL

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(Received October 15, 1993)

(Accepted January 24, 1994)

The physicochemical pretreatment of ball milled rice straw with different oxidizing agents, peracetic acid, alkali-peroxide, manganese-peroxide compounds under steaming pressure were studied. The pretreatment resulted in major changes in chemical composition of rice straw. The peroxide treated substrates were found to be most susceptible to enzymatic saccharification. A maximum saccharification (77.4%) of alkaline-peroxide treated rice straw (5%, w/v) was achieved using cellulase enzyme produced by mixed cultivation of *Trichoderma reesei* Rut C-30 and *Aspergillus ochraceus* containing 1.83 FPU, 1.63 cellobiase and xylanase 2.03 IU/ml. The hydrolysate was fermented using coculture of a temperature resistant strain of *Saccharomyces cerevisiae* and *Pachysolen tannophilus* resulting in 1.5% (w/v) ethanol. The SSF of 10.0% (w/v) H_2O_2 - $MnSO_4$ treated straw yielded maximum ethanol (2.9%, w/v) after 72 h at 40 °C. As a consequence of the well-balanced cellulase production by mixed fungal culture, the supplementation of cellobiase or xylanase was not necessary in the simultaneous saccharification and fermentation process.

The suitability of bioconversion of rice straw into ethanol is well recognized [1, 2]. The bioavailability of both pentoses and hexoses in the agroresidue makes it desirable to utilize both sugars into ethanol which is an important step for making the process economically viable [3]. Yeast strains of *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis* have been documented to ferment five carbon sugars efficiently [4]. Therefore, Simultaneous Saccharification and Fermentation (SSF) process, a potential method for obtaining ethanol from cellulose which is being used in Natick process for conversion of municipal solid waste (MSW) into ethanol [5] was

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employed in the present studies. However, the Natick process fails to utilize hemicellulosic fraction for bioconversion. Therefore, the present investigation reports SSF of rice straw using balanced cellulase enzyme complex including xylanases and yeast coculture of *Saccharomyces cerevisiae* and *P. tannophilus* for complete bioconversion of rice straw sugars (hexoses and pentoses) into ethanol.

Materials and methods

The rice straw was subjected to ball milling (2 mm particle size) prior to pretreatment, than 10.0 g aliquots were suspended in 200 ml volumes of (a) NaOH, 1.0%; (b) alkaline H₂O₂ 1.0%, pH adjusted to 11.5 using NaOH [6]; (c) hydrogen peroxide solution containing MnSO₄/MnCl₂ at a concentration of molar ratio 1:100 against H₂O₂ [7]; (d) peracetic acid, 1.0%; (e) distilled water.

The samples were subjected to autoclaving at 20 psi pressure for 30 min for autohydrolysis and steam was released immediately, thereafter. The pretreated samples were filtered, washed and oven dried at 60 °C to constant weight. The dried samples were then analysed for their composition i.e. cellulose, hemicellulose and lignin content.

Cellulase production. The cellulase enzyme was produced by mixed cultivation of *T. reesei* RUT C-30 and *A. ochraceus* in basal medium containing 6.0% (w/v) ball milled rice straw as carbon source [8]. The crude enzyme preparation had the following enzyme activities (IU/ml); FPU (filter paper units) 1.83; cellobiase 1.63; and xylanase 2.03.

Enzyme activity. The enzyme activity was expressed in IU. One IU was defined as micromoles of glucose or xylose released per ml of enzyme per minute.

Yeast inocula for fermentation. A yeast strain of *S. cerevisiae* obtained from Jagatjit Distilleries, Kapurthala (India) and *P. tannophilus* NRRL 4160-Y was kindly provided by Dr. Kurtzmann, NRRL, Illinois, USA. These cultures were grown and maintained on GYE and XYE medium, respectively [9].

Saccharification and fermentation. The pretreated rice straw (5.0% dry wt. basis) was saccharified at 40 °C for 48 h using crude enzyme extract containing 183 FPU, 163 IU cellobiase and xylanase 203 IU per 100 ml of crude enzyme. The saccharification was carried out in 250 ml conical flasks on water bath shaker at 100 rpm. The hydrolysate after aseptic centrifugation was supplemented with fermentation medium components described earlier [10], except sugar was replaced by hydrolysate sugar syrup. It was sterilized and subjected to fermentation in 250 ml conical flask by using a yeast coculture *P. tannophilus* + *S. cerevisiae* at 2.0 and 8.0% (v/v), respectively; incubation lasted for 48 h at 40 °C. The Simultaneous Saccharification and Fermentation (SSF) was carried out using 5.0 and 10.0% (w/v) pretreated rice straw alongwith crude enzyme and yeast coculture for 72 h at 40 °C, as described above.

Analytical methods. The cellulose, hemicellulose and lignin were analysed by the method of Goering & VanSoest [11]. The cellulase activity was assayed using filter paper as substrate [12]. Cellobiase activity was estimated using 1.0% cellobiose as substrate [13] and xylanase activity was determined by release of xylose from a 1.0% (w/v) xylan solution [14]. The reducing sugars were estimated by DNS method [15]. Ethanol was determined by gas liquid chromatography with chromosorb-102 column [16].

Results

Effect of different oxidizing agents on pretreatment of rice straw. The pretreatment of rice straw with any of the tested oxidizing agent resulted in solubilization of cellulose, hemicellulose and lignin fractions of the substrate. This is

evident from the change in the composition of substrate after pretreatment as recorded in Table I. The treatment with peroxide system such as alkaline H_2O_2 , H_2O_2 -Mn system, peracetic acid or NaOH resulted in increased amount of holocellulose as compared with the untreated control. The maximum holocellulose content was found in peracetate treated substrate (89.3%) followed by H_2O_2 alkaline and H_2O_2 -MnSO₄ system (Table I).

Table I
Chemical analysis of pretreated rice straw

S. No.	Treatment	Grams	Per cent, w/v			
		Net wt. recovered	Cellulose	Hemicellulose	Holocellulose	Lignin
1	Control	100.0	39.2	30.0	69.2	9.6
2	NaOH	55.4	52.0 (28.8)	30.6 (16.9)	82.6 (45.7)	7.0 (3.9)
3	H_2O_2 -Alk	51.2	51.7 (26.5)	35.4 (18.1)	87.1 (44.6)	4.8 (2.5)
4	H_2O_2 -MnCl ₂	79.0	50.0 (38.5)	32.7 (25.4)	82.7 (64.3)	3.6 (2.9)
5	H_2O_2 -MnSO ₄	74.0	50.4 (37.3)	36.4 (26.9)	86.4 (64.2)	3.8 (2.8)
6	Peracetate	59.0	54.5 (32.2)	34.9 (20.6)	89.3 (52.7)	5.6 (3.3)
7	Steam	71.0	38.1 (27.1)	30.0 (21.3)	68.1 (48.3)	9.2 (6.5)

Alk: Alkaline

Treatment conditions: 15 psi, 121 °C for 30 min

The values in parentheses indicate the unsolubilized portion in per cent

Enzymatic saccharification and subsequent fermentation. The peroxide treated substrates showed higher susceptibility to the enzymatic treatment using cellulase obtained from mixed cultivation of *T. reesei* and *A. ochraceus* a hyper cellobiase producing strain. The crude cellulase enzyme mixture was well balanced and each 100 ml of enzyme in which the substrate (5%, w/v) was suspended contained 183 IU FPU, 163 IU cellobiase and 203 IU xylanase. The maximum extent of saccharification was achieved in alkaline- H_2O_2 treated rice straw (77.4%). The released sugars (3.7%, w/v) were subsequently fermented with a coculture of *P. tannophilus* and *S. cerevisiae* to an ethanol content (1.5%, w/v).

Simultaneous saccharification and fermentation. The SSF of peroxide treated rice straw was carried out at 40 °C for 72 h. The alkaline H_2O_2 treated rice straw (substrate concentration of 5%, w/v) yielded 1.7% (w/v) ethanol during SSF process.

However, the H_2O_2 - MnSO_4 treated rice straw (substrate concentration of 10%, w/v) resulted in maximum ethanol content (2.9%, w/v) during SSF process.

Discussion

The Natick process uses a technically feasible SSF approach for the conversion of MSW that contains cellulose (57%) into ethanol [5]. The peroxide treated rice straw contained higher holocellulose content, since, the peroxide system brings about neither removal of cellulose nor lignin but causes loosening of intermolecular linkages between lignin and cellulose [6, 7] thus resulting in higher susceptibility to the lignocellulolytic enzyme action as found during saccharification and subsequent fermentation experiment (Table II).

The crude cellulase enzyme complex used in the present studies was produced by a mixed cultivation of *T. reesei* Rut C-30 and *A. ochraceus* [8]. The produced enzyme complex was balanced and contained high titre(s) of filter paper activity, cellobiase and xylanase and therefore, no supplementation of β -glucosidase and xylanase in the reaction was necessary, moreover, the enzyme was effective for saccharifying the peroxide treated rice straw with high holocellulose content into reducing sugars. However, Szczodrak [17] and Arai et al. [2] have reported the addition of β -glucosidase produced by *A. niger* for improving the ethanol yield during SSF of wheat straw and rice straw, respectively. In the SSF process being reported here a yeast coculture of *P. tannophilus* and a thermotolerant strain of *S. cerevisiae* was employed for fermentation of mixed sugars [10] that resulted into ethanol yield comparable that achieved by Szczodrak [17].

Table II

Saccharification and subsequent fermentation of pretreated rice straw

S. No.	Treatment	Carbohydrate content (g)	Total sugars released (g)	Saccharification (%)	Ethanol % (w/v)	Y = P/S
1	Untreated	3.4	1.1	29.1	0.3	0.52
2	NaOH	4.1	2.5	54.9	0.9	0.70
3	H_2O_2 -Alk	4.3	3.7	77.4	1.5	0.79
4	H_2O_2 - MnCl_2	4.1	3.5	76.8	1.4	0.78
5	H_2O_2 - MnSO_4	4.3	3.4	75.3	1.4	0.76
6	Peracetic acid	4.5	3.1	62.0	1.3	0.70
7	Steam	3.4	1.8	47.6	0.6	0.65

Alk: Alkaline

Substrate conc. 5% (w/v)

Table III

SSF of pretreated rice straw

S. No.	Treatment	Ethanol* (% w/v)	Ethanol** (% w/v)
1	H ₂ O ₂ -Alk	1.7	2.6
2	H ₂ O ₂ -MnCl ₂	1.6	2.8
3	H ₂ O ₂ -MnSO ₄	1.6	2.9
4	Peracetate	1.3	2.2

Alk: Alkaline

*5% w/v and **10% w/v pretreated substrate working vol. 100 ml;
temp. 40 °C; agitation: 150 rpm; incubation period: 72 h

Therefore, in order to further economize the SSF process, the use of a mixed culture cultivation for balanced enzyme production and the yeast strain capable of metabolizing C₅ and C₆ sugars present in the hydrolysate is of prime importance for making the bioconversion of lignocellulosics into ethanol a commercially viable process [3].

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A SIMPLE AND RAPID SEMIQUANTITATIVE METHOD FOR MEASURING CELLULASE ACTIVITY IN AGAR MEDIA

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(Received November 1, 1993)

(Accepted February 17, 1994)

A simple and rapid semiquantitative technique for the determination of fungal cellulolytic activities in solid (agar slant) media has been developed. This method is a combination of Congo-red staining widely used for qualitative cellulase detection and common cellulase activity tests. Previous investigation on the adsorptive effect of cellulose content of media showed that the real enzyme activity values can be measured with minimum loss by means of agar discs cut from the most active zones of slants visualized by Congo-red staining. Different cellulase activity tests (FPase, CMCase and β -glucosidase by PNPG-method) of seven cellulolytic fungal strains were investigated by this technique. Data give information on the different enzyme profiles of the species. The method can be regarded as very simple and suitable for simultaneous rapid comparison of cellulase components of greater series of fungal strains from agar slant cultures. It can also be used in the case of fungi unable to grow in liquid cultures.

The application of cellulolytic fungi for industrial purposes has an increasing significance. Therefore reliable and rapid methods for measuring cellulase activity are essential. Commonly used techniques [1], are suitable for measurement of activity from liquid samples only. Viscosimetry [2], photometry based on hydrolysis of stained filter paper [3], or HPLC methods [4, 5] giving more precise results than the former ones are based on liquid cultures as well. A number of fungi (mainly basidiomycetes) are not able to grow in liquids. They require solid surface for adherence of the mycelium by all means. Qualitative detection of cellulolytic activity from agar slants is feasible [6–9], but so far no quantitative methods have been processed for activity measuring in solid media. (The use of liquid extracts from solid media gives false results, as enzymes are adsorbed strongly on their substrates and so concentrations are often not in agreement with the original.)

In this paper we recommend a simple and rapid method suitable for quantitative comparison in portions and changes of cellulase enzyme profiles in greater series of fungal strains is suggested. It is based on combining Congo-red staining with standard

cellulase activity methods (FPase, CMCase, PNPG method). The experiment can be carried out with only one agar slant culture.

Seven cellulolytic fungal strains were examined. The cellulase enzymes of some of these species are much investigated and widely used in the industry while others are active wood rotting fungi with entirely unknown cellulase systems.

Materials and methods

Strains investigated. The following fungal strains, obtained from the Botanical Department of Hungarian Natural History Museum were used in our experiments: *Aspergillus flavus* (M₇₆), *Lentinus edodes* (M₉₄), *Serpula lacrymans* (M₁₁₆), *Omphalotus olearius* (M₁₂₉), *Phanerochaete chrysosporium* (ATCC₃₂₆₂₉), *Pholiota squarrosa* (M₁₃₅), *Trichoderma harzianum* (WT₈₇₀₈).

Media and activity testing. Investigations were carried out on Czapek-Dox agar slants containing 1% w/v CMC (carboxymethyl cellulose Na-salt, Serva) or microcrystalline cellulose (Avicel, Serva) instead of sucrose as carbon source and 2% w/v Difco agar. For calibration of the recovery of cellulase from agar media Onozuka R 10 (Serva) enzyme standard was used. Agar slants were stained with CCB (Coomassie Brilliant Blue R-250, Reanal) for protein [10] and with Congo-red (Reanal) [9] cellulase activity by methods described elsewhere [8, 11].

The experiment was carried out in the following steps.

(1) First the recovery of cellulase from agar medium was measured. Different agar slants were prepared in 90 mm diameter Petri dishes, as follows: (a) CMC 10 mg/ml, cellulase 0.1 mg/ml, agar 20 mg/ml; (b) Avicel 10 mg/ml, cellulase 0.1 mg/ml, agar 20 mg/ml; (c) cellulase 0.1 mg/ml, agar 20 mg/ml; control: 0.1 mg/ml cellulase solution in distilled water.

(2) For the determination of cellulase activity of fungal strains CMC-slants were inoculated using mycelial discs 6 mm in diameter cut from slant cultures maintained on malt agar. On the 5-7th days, when the culture reached 35-70 mm diameter, the slants were cut into four segments. Two of them were stained separately in Petri dishes with CCB and Congo-red. Staining developed places of the slant with the highest cellulase activity (with Congo-red staining clear halo, with CCB dark blue arc). (See Fig. 1.) Discs 5 mm in diameter were cut from the active places of the intact segments using glass tubes, after having removed the mycelium. These discs (average volume 0.25 ml) were used in activity tests. Experiments were carried out in five parallels.

(3) The following enzyme activities were determined from disc samples: (a) activity of the whole cellulase complex by FPase test with DNS reagent; (b) endoglucanase activity based on sugar concentration liberated from CMC using DNS reagent; (c) β -glucosidase activity measured by PNPG test. All these methods were used according to the techniques of Ghose et al. [1].

Results and discussion

Exact quantitative determination of cellulase activity cannot be realized perfectly due to a number of influencing factors. Cellulase itself is not a well-defined term as it is a synergistic complex that consists of enzymes differing in quality, proportion and environmental sensitivity. Therefore appropriate techniques suitable to draw quantitative comparison between cellulolytic ability of different fungi are practically profitable without being precisely quantitative.

The aim of the recent paper was to process a simple and rapid semiquantitative method to determine cellulase activity of fungi growing in solid agar media. Widely used Congo-red staining was combined with common cellulase activity tests.

The first question was the possibility of measuring the activity of cellulase excreted into agar medium with liquidphase cellulase tests (e.g. FPase) by means of inserting the enzyme with agar discs cut from solid media instead of culture filtrate. Enzymes may partly be adsorbed in agar media and therefore lower activities can be detected. This is especially significant in the case of cellulose containing media since cellulases are strongly adsorbed on their substrates [12].

Primarily the possibility of recovery of cellulase from agar media and its proportions were examined. Results are shown in Table I. Data show that in spite of adsorption enzyme activity values can be measured in agar discs quite unalteredly. Highest decrease of activity can be seen in the case of PNPG test.

Table I

Effect of agar media on cellulase activity measurable from solid phase containing 0.1 mg/ml enzyme

Medium	Cellulase activity ($\times 10^{-2}$ U/ml)		
	β -glucosidase	FPase	CMCase
Control solution	4.9 $\pm$ 0.21	3.7 $\pm$ 0.25	5.9 $\pm$ 0.35
CMC-agar	3.8 $\pm$ 0.08	0.8 $\pm$ 0.11	3.4 $\pm$ 0.19
Avicel-agar	3.2 $\pm$ 0.13	3.1 $\pm$ 0.17	4.9 $\pm$ 0.25
Water-agar	2.5 $\pm$ 0.11	3.5 $\pm$ 0.09	5.1 $\pm$ 0.40

The best reproductibility is given by CMCase test on water-agar. On the basis of these results the conclusion can be drawn that using agar disc samples taken from the parts of CMC medium showing maximum enzyme activities where digestion of cellulose is perfect (the clear zones visualized by Congo-red staining), cellulase activities can be determined free of adsorptive effects and data will be near the original values.

Table II shows results of an experiment with seven cellulolytic fungi carried out in five parallels in the former way.

Data of Table II indicate well measurable and valuable results in the case of all fungal strains. Standard deviation values were considerable but never reached 50%. Error coming from the repeated use of the method was determined by an extra model experiment using 0.5 mg/ml glucose containing agar discs in nine parallels and turned out to be below 1%. Main error is originated from the unequal dispersion of enzyme and cellulose in the agar slants and discs. Mycelial fragments adhering to disc surface (although they are required to be removed) can cause further error. Sugar liberated from cellulose may also influence enzyme activity by feed back inhibition.

Table II

Cellulase activities of seven fungal strains measured by the suggested method from CMC containing Czapek-medium

Fungus	Cellulase activity ($\times 10^{-2}$ U/ml)		
	β -glucosidase	FPase	CMCase
<i>A. flavus</i>	4.7 $\pm$ 1.45	0.2 $\pm$ 0.02	4.0 $\pm$ 0.74
<i>L. edodes</i>	3.5 $\pm$ 0.95	3.6 $\pm$ 0.10	5.3 $\pm$ 0.36
<i>O. olearius</i>	3.2 $\pm$ 0.75	0.8 $\pm$ 0.36	0.7 $\pm$ 0.10
<i>P. chrysosporium</i>	0.9 $\pm$ 0.65	0.3 $\pm$ 0.10	3.5 $\pm$ 0.00
<i>P. squarrosa</i>	0.3 $\pm$ 0.03	1.8 $\pm$ 0.31	1.6 $\pm$ 0.80
<i>S. lacrymans</i>	4.4 $\pm$ 0.05	1.0 $\pm$ 0.15	2.8 $\pm$ 0.16
<i>T. harzianum</i>	6.4 $\pm$ 1.55	0.5 $\pm$ 0.12	1.5 $\pm$ 0.90

Cellulolytic activity of the investigated strains was quite different. An altering pattern of enzyme types was seen as well. High FPase and CMCase activity could be measured in the case of wood-rotting fungi (e.g. *L. edodes*, *S. lacrymans*, *P. squarrosa*). β -glucosidase activities of *A. flavus* and *T. harzianum* were higher than FPase. Naturally these proportions can vary in different media and liquid cultures.

Summing up, the method introduced here can be evaluated as an appropriate semiquantitative technique for comparing cellulase enzyme activities of different fungi in agar slant cultures.

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HLA ASSOCIATED CYTOTOXIC ANTIBODY PRODUCTION IN DIALYZED CHRONIC URAEMIC PATIENTS

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(Received November 3, 1993)

(Accepted January 3, 1994)

We studied the level of the cytotoxic anti-HLA antibodies (CTA) in 90 hemodialyzed chronic uraemic patients who were on waiting list of transplantation. An association between alleles of HLA A1, B8, DR3 haplotype and the level of CTA was studied. The ratio of the reduced CTA producing subjects was significantly higher in the HLA B8 and DR3 carriers. We could observe the highest ratio in the joint appearance of these alleles. In the presence of HLA A1 allele the ratio was decreased beside B8 or DR3 even as joined attendance of these alleles. Our data suggest a different role of these linked antigens in the regulation of cytotoxic antibody production.

The HLA B8 and/or DR3 antigens, which are frequently inherited linked with HLA A1 antigen [1] are associated in Caucasians with several immune-mediated diseases and decreased immune-responsiveness – low antibody production [2, 3, 4] and defective FcReceptor functions [5] as previous reports showed. There is evidence that bearers of these antigens may display significant changes in immune parameters when compared to individuals not having these antigens [6]. These changes may reflect the action of gene(s) in linkage disequilibrium with these alleles, which confers immunodeficiencies [7].

Monitoring of cytotoxic anti-HLA antibodies in prospective recipients of cadaver kidney transplants could be an important additional parameter for the determination of the prognosis of immune response following transplantation. We have examined association between serum level of cytotoxic anti-HLA antibodies and particularly studied phenotypes.

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Material and methods

Subjects. The study consisted of 90 Caucasian prospective recipients of cadaver kidney transplants, who were on waiting list of transplantation and screened for circulating cytotoxic anti-HLA antibodies (CTA) in average monthly, over periods ranging between 1 and 2 years. Twenty-nine out of the 43 females and 47 males have previously received in average 800 (± 400) cm³ blood. The mean age was 37 (± 16.3) years. They have 1880 test results after the studied interval. The patients were selected to eight different groups on the basis of their HLA phenotypes: A1 (35), B8 (23), DR3 (26), A1-B8 (19), A1-DR3 (6), B8-DR3 (4) and A1-B8-DR3 (13).

HLA typing. HLA A, B, C and DR typing was carried out by the standard NIH microlymphocytotoxicity test [8].

CTA level determination. Serum was collected from each patient for total periods ranging between 1 and 2 years. Each serum was screened on a 60-member frozen peripheral blood lymphocyte panel by the standard lymphocytotoxicity test. The cell destruction was greater than 30% in CTA positive cases and less than 30% in the negative ones.

Statistical analysis. Statistical analysis of data was performed using a suitable computer program. To analyze the studied population of the exposed subjects we also considered the F test for heterogeneity. To detect the existence of an association between CTA production and HLA alleles, an examination of independence was employed by the Chi Square (χ^2 , Yate) test.

Table I

Results of the examination of independence between different HLA phenotypes and reduced cytotoxic anti-HLA antibody production

HLA phenotype	χ^2	Level of significance
A1	1.42	$p \leq 0.2$
B8	3.75	$p \leq 0.05^*$
DR3	6.83	$p \leq 0.01^{**}$
A1, B8	1.90	$p \leq 0.17$
A1, DR3	16.37	$p \leq 0.001^{***}$
B8, DR3	46.40	$p \leq 0.001^{***}$
A1, B8, DR3	0.42	$p \leq 0.5$

*Significant association

Results

There were no significant differences between the pooled groups from aspects of sex, age, number of dialyses and the volume of previously getting blood transfusions. We have found a significant association between the HLA phenotype and the level of CTA production in the following groups (in ascending order of correlation): HLA B8; DR3; A1, DR3; B8, DR3. HLA A1 allele could not associate to reduced CTA production neither alone nor joined with the HLA B8 allele, and even the A1, B8, DR3 haplotype has not shown association with CTA level (Table I). The

"other" phenotypes (on which no special emphasis was laid) also have not shown any association with the CTA production. Table II displays the percentage distributions of CTA negative and positive cases in different groups.

Table II

Percentage distributions of CTA negative and positive cases in different HLA phenotypes

HLA type	(%)	Positiv	(%)	Negativ	(%)	Σ
A1	3 (38.9)	46 (61.5)		29 (38.5)		75
B8	2 (25.6)	27 (56.2)		21 (43.8)		49
DR3	2 (28.9)	31 (55.4)		25 (44.6)		56
A1, B8	1 (21.1)	25 (62.9)		15 (37.1)		40
A1, DR3	(6.7)	3 (23.3)		9 (76.7)		12
B8, DR3	(4.4)	2 (25.6)		6 (74.4)		9
A1, B8, DR3	1 (14.4)	16 (58.1)		11 (21.9)		27

N = number of subjects

Σn = number of examinations

Discussion

Our observations show that certain alleles of HLA A1, B8, DR3 phenotype (joined frequently with immunologic dysfunctions and autoimmune diseases) may not associate in an equal proportion with reduced cytotoxic antibody level in circulating blood. DR3 antigen shows the strongest association with decreased CTA level on the basis of examination of independence applying the Chi Square (Yate) test. The effect of B8 allele is not so strong, but can add to effect of DR3. In contrast, the HLA A1 allele which is frequently inherited linked with them, does not show any association with CTA level, neither alone nor joined with B8. Together with A1, B8 combination the DR3 also is not connectable to decreased level of cytotoxic anti-HLA antibodies. In light of this, HLA B8, DR3 positive subjects may be considered genetically to be low responders in the respect of antibody production. The known linkage disequilibrium of the A1 gene to B8 and DR3 suggest that the A1 gene, or other gene(s) which may be linked to this, can suppress the effect of B8, DR3 which causing immunologic dysfunctions. This findings suggest a possible evolutionary advantage of HLA A1, B8, DR3 haplotype which may be a reason of the linkage disequilibrium of these genes.

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DIVERSITY IN THE ANTIGENIC STRUCTURE OF DIFFERENT HEXON TYPES AMONG THE MEMBERS OF ADENOVIRUS SUBGENUS D

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(Received September 20, 1994)

(Accepted October 26, 1994)

Antigenic relationships of human adenovirus (Ad h) hexon types: 8, 9, 10, 13, 19, 26, 27 and intermediate type 9/13 (I13), belonging to subgenus D were studied with 61 hybridoma ascitic fluids containing monoclonal antibodies (MAbs). The MAbs were produced in three different panels directed against Ad h1, Ad h35, and bovine Ad type 2. The reactivity pattern (RP) and the degree of cross-reactivity (CR) of the MAbs were determined in indirect ELISA. By the 61 MAbs 8 different RPs could be distinguished. The distinct RPs of hexon types of 8, 9, 10, I13 and 19 were identical or nearly identical and the occurrence of the different eight RPs were based mainly on the combinations of positive or negative reactions of different MAbs with the hexon prototypes 13, 26 and 27. According to the different RPs and to the number of reacting and non-reacting MAbs with the different hexon types as well as on the base of the degree of CR three groups of hexon types with diverse antigenic structure could be distinguished. These data revealed the existence of remarkable diversity in the antigenic structure between type 27 and types 8, 9, 10, I13 and 19, as well as an intermediate position of prototypes 13 and 26.

The 49 human adenovirus types [1, 2] are being grouped to six subgenera (A-F) on the base of biological, immunological and biochemical characteristics. Different groups were identified based on haemagglutination properties and on serological reactions. Human adenoviruses have also been categorized according to their oncogenic and cell-transforming potential. Biological and serological activity are sometimes correlated with the percentage of guanine + cytosine in the DNA or with DNA homological relationships [3, 4]. Members of the same subgenus show similarity in these parameters.

Concerning the antigenic complexity of the adenovirus hexon, it was shown in our previous experiments that a large number of epitopes could be determined on the purified hexons by means of monoclonal antibodies. Besides the genus and type specific epitopes, most of the epitopes showed different intertype specificity. These epitopes were present on the surface of different hexon types in different intertype combinations, however their distribution did not coincide with the grouping of

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adenovirus serotypes into the current six subgenera [5–8]. It was also demonstrated that the antigenic relationship is close between the members of subgenus C and hexon types of subgenus D (8, 9, 10 and I13). In further experiments when additional serotypes of subgenus D were involved into the study of the cross-reactivity of several selected monoclonal antibodies remarkable differences were found between these hexon groups and the group of hexon types investigated earlier [9]. These results prompted us to examine the diversity and/or the similarity of the antigenic structure of adenovirus hexon types of subgenus D with a large number of hybridoma antibodies, raised against three different hexon types.

Materials and methods

Adenovirus hexon antigens. Viruses were propagated in HEp-2 or "293" cells [10] and the soluble hexon antigens were purified by repeated anion exchange chromatography and gel filtration [11, 12] and for human adenovirus type 1 (Ad h1) also by crystallization [13]. For the study of their antigenic structure purified hexons was prepared from 8 human adenoviruses of subgenus D. Types 9, 10, 13 (P13), 19, 26, 27 were prototype ones, type 8 was isolated and identified by us and a type 13/9 intermediate strain (I13) [14] was also included into the experiments. The protein content of purified hexons was determined by Lowry's method [15].

Preparation of monoclonal antibodies (Mabs). Hybridomas were produced by crystallized Ad h1, by purified Ad h35 hexons as described [16], while MAb directed against bovine adenovirus (Ad bos2) were kindly supplied by M. Rusvai. Ascitic fluids were prepared by injection of antibody-producing hybridoma cells into mice [16].

Indirect ELISA. Tests were carried out using 96-well polystyrene plates (Falcon Microtest II, T. C.). For coating 40 µg/ml concentration of purified hexon was used and MABs were added to the wells in serial twofold dilutions of ascites from 1:100. Detection of binding of MABs to the hexons was detected by goat anti-mouse immunoglobulin, labelled with horseradish peroxidase by the method already described [17]. The result were evaluated by a Medicor-Medilab EPD-1 photometer at 492 nm wavelength. The titres were expressed as $\log_2$ (reciprocal dilution of antibody $\times 10^{-2}$).

Results

Reactivity pattern of ascitic fluids. For the study of antigenic structure of eight different hexon types belonging to human adenovirus subgenus D sixty-one MAB-containing hybridoma ascitic fluids were used. The MABs were produced in three different panels directed against Ad h1, Ad h35, as well as against bovine Ad bos2. Table I shows the reactivity pattern (RP) of the 61 ascitic fluids with 8 hexon types of subgenus D and the specificity of the individual MABs or MAB groups determined in previous experiments [6, 18, 19, 23]. By seven individual MABs and eleven MAB groups containing 2 to 20 MABs eight different RPs could be distinguished, but in the reality more specific RPs should be calculated. The three genus specific RPs should mean one and the same epitope but the three type specific RPs should specify three different epitopes according to the 3 panels of MABs. As indicated in Table I, MAB 35H43 directed to Ad h35 showed the same RP as a five member group of MABs to Ad h1 while MAB 35H49 to Ad h35 showed the same RP as MAB 2C3 directed to Ad h1.

On the other hand MAbs in the second row in each of the three different panels of MAbs (first intertype specific row) MAbs reacted with all the hexon types of subgenus D tested but they gave no reactions with some different hexon types of subgenera other than subgenus D, therefore they should have different intertype specificity. The intertype specific RPs to types Ad h35 and Ad h7 should mean again an other epitope [24].

Table I

Reactivity pattern of 61 ascitic fluids with 8 human adenovirus hexon types belonging to subgenus D

Mab	Human hexon types of subgenus D								Specificity of MAbs ^c
	8	9	10	113 ^a	P13 ^b	19	26	27	
Directed to type: Ad h1									
1A3, 2C2	+	+	+	+	+	+	+	+	genus
H12, 5A4, 5B4	+	+	+	+	+	+	+	+	intertype
5A1	+	+	+	+	+	+	—	+	intertype
1A2, 1A6, 1B1, 1B5, 1B6, 1C4, 1C5, 1D4, 1D6, 2A2, 2A3, 2A4, 2A5, 2B1, 2B2, 2B3, 2C4, 2C5, 2D1, 2D2	+	+	+	+	+	+	+	—	intertype
2A1, 2A6, 2D6	+	+	+	+	—	+	+	—	intertype
2C3	+	+	+	+	+	+	—	—	intertype
1A5, 1B2, 2B5, 2C1, 2C6	+	+	+	+	—	+	—	—	intertype
5C1, 5D1, 5D2	—	—	—	—	—	—	—	—	type to Ad h1
Directed to type: Ad h35									
35H15	+	+	+	+	+	+	+	+	genus
35H26, 35H50	+	+	+	+	+	+	+	+	intertype
35H49	+	+	+	+	+	+	—	—	intertype
35H43	+	+	+	+	—	+	—	—	intertype
35H18, 35H54, 35H55, 35H56, 35H57, 35H58, 35H59, 35H62, 35H63	—	—	—	—	—	—	—	—	intertype to Ad h35 and Ad h7
35H10, 35H30, 35H51	—	—	—	—	—	—	—	—	type to Ad H35
Directed to type: Ad bos2									
IVF3	+	+	+	+	+	+	+	+	genus
B11	+	+	+	+	+	+	+	+	intertype
A12, CA12	—	—	—	—	+	+	+	+	intertype
IIA9, IVF5	—	—	—	—	—	—	—	—	type to Ad bos
Number of reacting/ nonreacting MAbs	42/19	42/19	42/19	42/19	35/26	44/17	35/26	13/48	

^aI13: intermedier type 13/9

^bP13: prototype Ad h13

^cGenus, intertype and type specificities were determined in previous experiments [9, 23, 24]

According to the different RPs and to the number of reacting and non-reacting MABs three groups of hexon types with diverse antigenic structure seem possible to be distinguished. Table I shows that the RPs of hexon types 8, 9, 10, I13 and type 19 are identical. The only difference is that type 19 reacted with two additional MABs than the former four. The ratio of the reacting and non reacting MABs from the 61 MABs in the case of hexon type 19 are 44/17, and in the case of types 8, 9, 10, and I13 are 42/19. This phenomenon reflects a strong similarity in the antigenic structure of this group of hexon types. On the base of the antigenic structure the second group of hexons could be hexon types P13 and 26. The numbers of the reacting and non-reacting MABs in the case of both types are 35/26 but in contrast to the former group that does not mean complete similarity in the RPs because six MABs (5A1, 2A1, 2A6, 2D6, 2C3, 35H49) reacted differently with this two hexon types. The third type which could be distinguished is hexon type 27. The numbers of the reacting and non-reacting MABs with this type are 13/48. This means that besides the four MABs with genus specificity only 9 MABs gave cross-reaction with this hexon type indicating the existence of remarkable differences in the antigenic structure between type 27 on the one hand and types 8, 9, 10, I13 and 19 on the other hand. Types P13 and 26 probably could have an intermediate antigenic structure between the two groups. From Table I it can be clearly seen that for the evaluation of the diversity among the hexon types those MABs cannot be used which reacted uniformly with none of the hexon types of subgenus D, i.e. the 8 type specific MABs and the 9 intertype specific MABs to type 35 and to type 7. The existence of different RPs is based mainly on the combinations of positive and negative reactions of the different MABs with the hexon types P13, 26 and 27.

Degree of the cross-reactivity. For the determination of the degree of cross-reactivity the titration of all the MABs were carried out with all hexon types in serial twofold dilutions of the ascitic fluids from the dilution of 1:200 at first, and the titres were expressed as $\log_2$ (reciprocal dilution of antibody $\times 10^{-2}$) thus a titre of 1 means a dilution of 1:200 and a titre of 10 – which often occurred – means a dilution of 1:102400. MABs showing lower titre than 1 were retitrated from the dilution of 1:30 and were considered positive from the titre of 0.3 (1:120). The degree of cross-reactivity was calculated from the titres as used by Yewdell and Gerhard [20] with the help of the formula:

$$\text{degree of CR} = \log_2 \left(\frac{\text{titre with heterologous hexon}}{\text{titre with homologous hexon}} \times 100 \right)$$

The homologous hexon types in the case of the three panels of the MABs were Ad h1, Ad h35 and Ad bos2, respectively, and according to the formula the degree of CR is 6.6 for the homologous hexon types. Comparing to this value the degree of CR of every individual MAB with a given hexon type their degree of CR have been considered high with a CR value of 6.6 or higher.

Table II

Degree of cross-reactivity (CR) of 3 panels of MAbs^a with 8 adenovirus hexon types of subgenus D

MAb	Human hexon types								Number of hexon types with 6.6 or higher CR
	8	9	10	I13	P13	19	26	27	
2C2	7.6	7.6	7.6	6.6	7.6	6.6	6.6	7.6	8
1A3	6.6	5.6	6.6	6.6	6.6	6.6	7.6	7.6	7
35H26	8.6	8.6	8.6	8.6	8.6	7.6	2.6	4.6	6
IVF3	6.6	6.6	6.6	6.6	6.6	6.6	4.6	4.6	6
35H15	9.6	9.6	9.6	8.6	4.6	8.6	4.6	3.6	5
H12	5.6	6.6	5.6	5.6	4.6	1.6	1.6	-0.4	1
5A4	4.6	4.6	4.6	5.6	0.6	4.6	3.6	3.6	-
5B4	4.6	4.6	5.6	5.6	-0.4	5.6	0.6	3.6	-
35H50	2.6	3.6	3.6	2.6	3.6	3.6	3.6	3.6	-
B11	1.6	0.6	0.6	0.6	1.6	0.6	1.6	3.6	-
5A1	6.6	5.6	5.6	5.6	-1.4	5.6		2.6	1
2A4	5.6	5.6	4.6	6.6	6.6	6.6	1.6		3
1D6	8.6	6.6	4.6	2.6	3.6	3.6	0.6		2
1C5	6.6	6.6	4.6	3.6	3.6	3.6	0.6		2
2D2	7.6	6.6	5.6	2.6	3.6	2.6	-0.4		2
2B3	7.6	6.6	5.6	4.6	5.6	4.6	0.6		2
1B5	6.6	6.6	6.6	3.6	3.6	2.6	-2.4		3
1A6	6.6	6.6	4.6	2.6	4.6	4.6	-0.4		2
2C4	6.6	6.6	4.6	4.6	5.6	4.6	-0.4		2
1A2	6.6	7.6	4.6	3.6	3.6	3.6	0.6		2
1B6	7.6	7.6	5.6	3.6	4.6	5.6	1.6		2
2A3	5.6	6.6	2.6	6.6	5.6	4.6	1.6		2
1B1	5.6	5.6	4.6	5.6	6.6	6.6	3.6		2
2C5	6.6	5.6	3.6	3.6	4.6	4.6	-1.4		1
2A2	6.6	5.6	5.6	3.6	3.6	2.6	-1.4		1
2B2	5.6	6.6	5.6	4.6	5.6	5.6	2.6		1
2D1	6.6	5.6	5.6	4.6	4.6	4.6	2.6		1
1C4	7.6	5.6	3.6	3.6	4.6	4.6	2.6		1
2B1	5.6	5.6	4.6	3.6	3.6	2.6	-0.4		-
2A5	4.6	4.6	2.6	4.6	4.6	4.6	2.6		-
1D4	4.6	5.6	3.6	5.6	5.6	4.6	1.6		-
2C3	6.6	6.6	5.6	3.6	4.6	2.6			2
35H49	5.6	5.6	4.6	4.6	3.6	7.6			1
2A1	6.6	6.6	5.6	5.6		4.6	6.6		3
2A6	6.6	6.6	5.6	3.6		2.6	2.6		2
2D6	5.6	5.6	4.6	5.6		2.6	3.6		-
35H43	7.6	7.6	6.6	6.6		6.6			5
1A5	7.6	7.6	5.6	3.6		6.6			3

Continued on p. 90

Table II continued

2C1	6.6	6.6	5.6	5.6		4.6			2
1B2	6.6	6.6	5.6	2.6		2.6			2
2B5	6.6	7.6	4.6	3.6		2.6			2
2C6	6.6	5.6	5.6	4.6		3.6			1
CA12					1.6	8.6	3.6	3.6	1
A12					4.6	6.6	5.6	5.6	1
Mean value of CR	6.34	6.30	5.30	5.03	4.31	4.66	2.14	3.31	
Number of MAb with 6.6 or higher CR values	28	24	7	8	6	12	3	2	

^aMAbs not reacting with any hexon type of subgenus D were not involved in this evaluation

MAbs giving no reaction with any hexon type of subgenus D were not involved in this evaluation. Thus the degree of CR was evaluated in the case of 44 MAbs. It can be seen from the data of Table II that with the exception of 8 MAbs, all other MAbs expressed high CR with one or more hexon types. High CR was found with one hexon type by 11 MAbs, with two types by 15, with three types by 4, with 5 types by 2, with six types by 2, with seven types by 1 and with all the eight hexon types by 1 MAb. Genus specific MAbs 1A3, 2C2, 35H15, and IVF3 have shown high CR values with all or most hexon types indicating the equally strong expression of the genus specific epitope on all types of hexons studied in subgenus D. Besides the genus specific MAbs only two other MAbs 35H26 and 35H43 have shown high CR values with 6 or 5 hexon types respectively. Table II shows also the number of the MAbs giving high CR, and the mean values of CR according the hexon types. These data confirm the existence of remarkable diversity in the antigenic structure between type 27 and types 8, 9, 10, I13 and 19 and the intermediate position of types P13 and 26. There are significant differences in the mean CR values and in the number of high CR MAbs in the case of types 27 and 26 and in that of types 8, 9, 10, I13 and 19. The number of high CR MAbs with the types 8 and 9 out of the reacting 42 MAbs are 28 and 24, respectively, with a 6.3 mean value of CR which indicates a closer antigenic relationship with each other than with the hexon types 10, I13, and 19 which have identical or nearly identical RPs with the types 8 and 9 but have shown high CR values only with 7, 8 and 12 MAbs, respectively, with a 5.3, 5.0 and 4.7 mean value of CR. In the intermediate group with type P13 only 6 MAbs and with type 26 only 3 MAbs showed high CR values in spite of the fact that the number of reacting MAbs were relatively high: 35 with both types. Type 26 have shown the lowest mean cross-reactivity value (2.1) among the hexon types. Type 27 the third type of antigenic structure, have shown a mean CR value of 3.3, and high cross-reactivity values were found only with 2 genus specific MAbs.

Discussion

Adenovirus serotypes are defined on the basis of their immunological distinctiveness as determined by quantitative neutralization (SN). If neutralization shows a certain degree of cross-reaction, distinctiveness of a new serotype can be assumed if its haemagglutinins are unrelated determined by haemagglutination inhibition test (HI) with polyclonal animal sera [3]. Members of subgenus D fit these criteria of distinctiveness, although strong cross-neutralizations, and by HI a great number of heterologous reactions were found, presumably due to a relationship of the type specific hexon antigen in SN and of the type specific fiber antigen in HI [21, 22]. Hierholzer and coworkers demonstrated HI cross-relationships among the so-called EKC subgroup of subgenus D, consisting of types 8, 9, 10, 19 and 37. It is noteworthy that almost only the members of this subgroup are pathogenic members of subgenus D besides the high numbered serotypes recently isolated and identified from AIDS patients [1, 22]. These results are consistent with our findings with MABs on purified hexons of types 8, 9, 10, I13, P13, 19, 26 and 27 of subgenus D. According to the number of reacting and non-reacting MABs as well as on the basis of different reactivity patterns and of the degree of cross-reactivity three groups of hexon types with diverse antigenic structure could be distinguished. It was demonstrated that the RPs of hexon types 8, 9, 10, I13 and 19 are identical or nearly identical showing a strong antigenic similarity among these hexon types. The data concerning the number of reacting and non-reacting MABs out of the 61 studied and the degree of cross-reactivity have shown a remarkable diversity in the antigenic structure of hexons between type 27 and types 8, 9, 10, I13 and 19 and the intermediate position of types P13 and 26. These significant differences concern mainly the presence or absence of the numerous intertype specific epitopes in different combinations on the surface of the individual hexon types. Subgenus D comprises the largest number of serotypes among all human subgenera. More than the half of all human serotypes belong to this subgenus. It could be expected therefore that diversities can be found in some parameters i.e. such as the intertype specific epitope composition of different hexon types. It is desirable, however, to extend the studies to more or all hexon types and to use even larger number of MABs and/or MABs directed to other hexon types, too, than in the present study. For the complete and exact determination of the epitope composition of the individual hexon types it would need to determine and define every individual intertype specific epitope with standard MABs of different panels and with the help of known and well-defined epitopes determine the epitope spectrum of the different hexon types.

Acknowledgements. The research program was supported by the grants No. 2612 and 6248 of the Hungarian Scientific Research Fund (OTKA) and by a grant of UNESCO-ROSTE No. 876.005.3. The authors are thankful to Dr. J. Facht and Dr. J. Erdei for contribution in preparing monoclonal antibodies. The skilled assistance of Mrs. Mária Sósuti and the help of Mrs. Valéria Kövesdi in the preparation of the manuscript is highly appreciated.

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**ANNUAL MEETING
OF THE HUNGARIAN SOCIETY
FOR MICROBIOLOGY**

**SZOLNOK, AUGUST 23–25, 1994
ABSTRACTS OF PAPERS AND POSTERS**

Symposium on Gastrointestinal Infections	95
Bacteriology	100
Virology and Immunology	102
Agricultural and Food Microbiology	111
Mycology and Industrial Microbiology	125

SYMPOSIUM ON THE CLINICAL, EPIDEMIOLOGICAL AND MICROBIOLOGICAL CHARACTERISTICS OF GASTROINTESTINAL INFECTIONS

A. SZALKA

Acute infectious diarrhoea from the clinician's point of view

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The differential diagnosis of acute infectious diarrhoea is expanding, as a number of diagnostic tests and therapeutic options are available. In view of this, many physicians find the evaluation and management of acute diarrhoea confusing. On a clinical basis, infectious diarrhoea can be divided into one of two syndromes, based on the pathogenic interaction of the infecting organism with the host intestine: 1. inflammatory, or bloody, diarrhoea; 2. noninflammatory, nonbloody diarrhoea. In general, inflammatory diarrhoeas represent the more severe spectrum of acute diarrhoeas and generally require more intensive treatment and evaluation. Noninflammatory diarrhoea is often a mild disorder but occasionally can cause severe volume depletion.

The goal of the approach to the patient with acute infectious diarrhoea is to distinguish medically important diarrhoea from benign self-limited illnesses. The approach is a clinical one and consists of careful work, focused on four pieces of information: 1. the severity of illness; 2. the duration of diarrhoea; 3. the epidemiologic setting in which the infection was acquired; 4. the competence of underlying host defence and immunity. Integration of these data allows an assessment of the need for specific diagnostic evaluation and therapy for the individual patient. It should be

emphasized that in the majority of cases, no diagnostic evaluation or specific therapy, other than volume repletion, is needed.

L. EMÓDY

**The role of tissue protein binding in the pathogenesis
of *Yersinia* infections**

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Three species of the genus *Yersinia* are pathogenic for humans: *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*. While *Y. pestis* is the causative agent of bubonic and pulmonary plague the other two pathogenic species cause enteral and extraintestinal manifestations being quite different from plague. It is a question why these genetically related bacteria are so different in causing clinical manifestations.

A virulence plasmid of 70 kilobase pair is characteristic for all the three species. This plasmid codes for several virulence related outer membrane proteins and secreted proteins being regulated by environmental conditions like temperature and calcium level. The same plasmid codes for YadA, the *Yersinia* adhesin determinant which enables bacteria to adhere to host cells, and resist to phagocytosis. Interestingly, *Y. pestis*, the most invasive species of the three fails to produce this protein because of a mutation at the relevant DNA sequence.

Beside the known functions of the YadA protein we have found a new function of this molecule. YadA enables bacteria to bind type I, type II and type IV collagens. These collagen types are major constituents of such important tissue components like connective tissue matrix, cartilage, and basement membranes. Adherence of bacteria to such tissue components might be involved in the process of colonization and invasion. Interactions of this type may also contribute to the development of well-known disease sequelae with connective tissue involvement.

Connective tissue binding capacity of *Y. enterocolitica* is entirely YadA dependent while *Y. pseudotuberculosis* is able to bind type IV collagen by an alternative mechanism encoded by chromosomal regions.

Though *Y. pestis* does not possess YadA mediated collagen binding mechanisms it is able to interact with type IV collagen through another surface protein. This protein is encoded by the 9.5 kilobase plasmid of *Y. pestis*, and is identical to a molecule with enzymatic activity, known as plasminogen activator. The same compound has been shown to mediate cell adherence and binding to laminin. This multifunctional molecule is a major factor of invasiveness of *Y. pestis* as the lack of it results in a millionfold increase of subcutaneous lethal dose for mice, while it has practically no influence on the intravenous lethal dose. In other words this virulence factor has got a pivotal role in destroying and penetrating tissue barriers. Binding to basement membrane proteins and subsequent desintegration of them seems a decisive step in this process. The above data also support the idea that acquisition of the 9.5

kilobase plasmid must have been an important step of the evolution of *Y. pestis* from *Y. pseudotuberculosis*.

É. CZIRÓK, M. HERPAY, I. GADÓ, J. PÁSZTI, G. V. LÁSZLÓ AND H. MILCH

**Enteropathogenicity of *Escherichia coli* –
controversial questions**

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Recent knowledge concerning the pathogenicity of enteropathogenic *Escherichia coli* (EPEC) was summarized and compared with those of our own results. Virulence markers (serogroup, adhesion to HEp-2 cells, enteroadhesive factors – EAF, local adherence – LA, diffuse adherence – DA, attaching and effacing factor – AECC), genetical background, proteins responsible for adherence, adhesion inhibiting factors, diagnostic use of fluorescein actin staining (FAS) and the newly recognized EPEC group (the enteroaggregative *E. coli* – EAggEC) were reviewed. Hungarian experiences regarding inhibition effect of lactoferrin on adherence of EPEC and occurrence of EAggEC (32 out of 311 strains were Clump positive) were reported. Colonization factors (CFAI, CFaII, E8775, PCFO159) and enterotoxins (heat labile-LT, heat stable-ST) of enterotoxin producing *E. coli* (ETEC) were analysed including experiences on their own isolates (15 and 28 strains were LT+ and ST+, respectively). On the basis of the above experiences ETEC can be imported and transmitted from person to person, thus toxin detection kits should be available in diagnostic laboratories in Hungary. Enterohaemorrhagic *E. coli* (EHEC) was characterized as etiological agent of haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP). EHEC caused epidemics of haemorrhagic colitis in the United States and Canada in the early 1980's, transmitted by undercooked meats; the isolates belonged to serogroup O157 and elaborated verotoxin (VT). Since that time EHEC occurred in most part of the world (UK, Japan, Europe etc.). On the basis of their occurrence in Hungary (23 out of 216 strains produced cytotoxin, 6 of them VT1 or VT2), methods for their detection (routine use of O157 antisera, Sorbitol MacConkey media, and verotoxin detecting tests – Denka Seiken product) should be introduced in Public Health Laboratories, and development of surveillance in cooperation with food, animal and human public health authorities seems to be advisable. Attention was drawn to the diagnostic difficulties of non-O157 VT+, O157 VT– *E. coli*, and non-*E. coli* VT+ strains.

T. PÁL

Application of new results concerning the pathogenesis of bacillary dysentery in prevention and diagnosis*Department of Microbiology, University Medical School, Pécs, Hungary*

Contrary to the former empirical approach, in the past 10–15 years a new strategy can be recognized in the development of vaccines and new diagnostic techniques. This strategy relies on the data obtained by studying the molecular biology, and the pathogenesis of various infections. Knowing the microbial virulence factors and their roles allows us to design vaccines inducing an immune response effectively blocking key steps of the pathogenesis without causing harmful side effects. Diagnostic methods developed according to the same principles are highly specific because they detect genes or gene products distinguishing the pathogen from non-pathogenic strains of similar, or even identical species. This tendency of research can also recently be observed in studying *shigellae* and enteroinvasive *Escherichia coli* (EIEC) strains, i.e. the etiologic agents of bacillary dysentery. It has been known since the sixties that virulent *Shigella* strains can infect the intestinal epithelial cells. However, only in the past 10–12 years – mainly due to the molecular biological approach – we have started to understand the details of the bacterium–host cell interactions. The co-ordinated expression of chromosomal and extra-chromosomal genes located on a 220 kb plasmid is necessary for the invasion of the epithelial cells, for lysing the phagosomal membrane, and for the actin-based intra- and intercellular spread of the bacterium. There are several promising vaccine candidate strains attenuated by mutations induced in some of these virulence-related genes. These strains – though avirulent – kept their immunogenicity, and their invasive capacity thought to be necessary for the optimal antigenic stimulus. Such experimental vaccines for example are those with mutations rendering the bacteria to be unable to spread intra- and intercellularly, disconnecting them from the environmental osmotic signals, or preventing their intracellular growth. Recently, the introduction of DNA probes and PCR systems specific to virulence-related genes or DNA sequences of *shigellae* have improved the diagnostic possibilities of the routine laboratories. A promising alternative for these molecular biological techniques could be the immunodetection of virulence-specific antigens coded by the invasion plasmid of *shigellae* and EIEC strains. In the lecture the experimental vaccines and diagnostic tools developed according to this virulence factor-oriented approach will be briefly reviewed.

I. GADÓ, J. PÁSZTI, V. G. LÁSZLÓ, B. NAGY, I. BARCS and H. MILCH

Connection among hybridization of *Salmonella enteritidis* strains with STb and EIEC probes, small plasmid carrier state and phage-types

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A total of 197 *Salmonella* strains belonging to 26 different serotypes, originated from human, animal and food sources were hybridized *in situ* with gene probes suitable for detection STb enterotoxin gene of *E. coli* and the invasivity genes for detection of EIEC and *Shigella* strains. The former one was a fragment of size 460 bp of *E. coli* origin (described by Lee et al. 1983), the latter being originated from *S. flexneri*, the size of fragments being 17 and 7.6 kb (Boileau et al. 1984). Out of the 87 examined *S. enteritidis* strains, plasmids smaller than 5 Md were carried by 51 (~60%). These plasmids, except two, hybridized with the above probes. It was proved by Southern-blot technics that this small plasmids were responsible for the hybridization. A good correlation was found between the small plasmid carrier state (hybridization with the probes) and phage restriction to the two Spanish phages, which were used to subdivide phage-types 1 and 6 of *S. enteritidis*. Phage-types 1b, 1c and 6b, 6c, resp., were developed as a consequence of phage restriction to these two phages. Phage restriction occurred among strains belonging to other serotypes, when they harboured hybridizing small plasmids. The rate of *S. enteritidis* strains of phage-type 6 increased in the last two years in Hungary and also the rate of those strains within this phage-type which restricted the Spanish phages. Among other not frequent human serotypes the number of hybridizing strains was much smaller and the correlation was not so strict between hybridization and small plasmid carrier state.

GY. SZÜCS, M. ÚJ, I. MIHÁLY, J. DEÁK, I. TÓTH and A. KÁTAI

Viral gastroenteritis: viruses, laboratory diagnosis – investigations in Hungary

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The authors review the available information and attempt to summarize the current position of the viruses commonly implicated in gastroenteritis, as well as they delineate the epidemiological importance of rotaviruses, enteric adenoviruses, and Norwalk virus in Hungary. Also, the available diagnostic methods and requirement for the more widely applicable diagnostic molecular techniques for viruses considered as etiological agents in acute nonbacterial gastroenteritis are discussed. Since 1984 group

A human rotaviruses have been investigated by neutralizing monoclonal antibodies specific for serotypes G1, G2, G3, and G4 in an enzyme immunoassay in two collections of stool specimens assembled from two geographic areas in Hungary. Rotaviruses were detected in 27% and 29%, respectively, of the samples with the highest rate of positivity in the first months of each year. Majority of them was typed as follows: G1 (81%), G2 (4%), G3 (1%), G4 (5%), or mixed type (1%). G1 was the predominant type in all rotavirus seasons with exception in two years when serotypes G2 and G4 predominated. Analysing the genom, different electropherotypes were detected with a shift of the predominant G1 types each 2 to 3 years. Electropherotyping made also possible to reveal nosocomial infections and three group C rotavirus strains. (Group B rotaviruses have not been detected yet.) Detection and genom analysis of the enteric adenoviruses was successful first in 1989 in Hungary. All isolates were characterized as type 41. Using latex agglutination, monoclonal antibodies in ELISA, "293" cell cultures, and restriction enzyme digestion of the isolated viral DNA, more strains were found but no type 40. However, among types 41 two genotypes were observed. Without seasonality enteric adenoviruses were detected in 3% to 7% of samples collected from children under five years with acute diarrhea. In 1993, with the help of American scientists, experiments were started to reveal the presence of Norwalk virus in the Hungarian population. Using recombinant Norwalk virus antigen in an ELISA system, 61% of the 1030 serum samples from 13 age groups contained antibodies. Up to 20 year of age about 80% of population has developed antibodies against this virus. Recently, tests are also planned for detection of astroviruses in collected stool samples. Laboratory diagnostic methods are discussed, emphasizing the increasing applicability of the PCR and other molecular techniques. Furthermore, the authors believe our knowledge will be much better within some years about the circulation and epidemiological role of the viruses implicated in gastroenteritis in consequence of the adaptation of molecular methods and of the cooperation among the virological laboratories, doctors, and epidemiologists.

BACTERIOLOGY

M. KERÉNYI, A. RITTER, G. BLUM, J. HACKER and L. EMÓDY

The influence of *leuX* locus on the virulence of uropathogenic *Escherichia coli*

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In the laboratory of one of us (J.H.) a transfer RNA locus, termed *leuX*, which codes for a leucine specific tRNA, was shown to be involved in the expression of a number of virulence associated genes in uropathogenic *Escherichia coli*. *leuX* mutants seem to be deficient in the in vitro expression of such important virulence associated

properties like production of type-I fimbriae, the capacity to grow in human serum, siderophore production, and motility. These in vitro results prompted us to perform virulence assays in mice to assess the in vivo role of the *leuX* locus in virulence. In our experiments the virulence of a *leuX* mutant of a urinary *E. coli* isolate was compared to that of its isogenic *leuX* positive recombinant when injected to mice intravenously. The results of these assays show that introduction of the *leuX* gene into the negative mutant significantly decreases the 50% lethal dose, and there is a highly significant difference in the death rates when a single dose of 2.5×10^8 bacterial cells is administered. There is a delayed blood and organ clearance for the *leuX* positive clone, and tissue tropism to the eye is expressed in the form of purulent endophthalmitis. Our results indicate that beside its well-known function of coding for a leucin specific tRNA the *leuX* locus exerts a marked influence on the in vivo and in vitro expression of several virulence associated genes. This global regulatory function of the *leuX* locus might perspectively be utilized in a general strategy to construct putative vaccine candidates.

L. FODOR, O. SZENCI, M. PETERS, J. VARGA, GY. SZEMERÉDI
and F. WYSZOCZKY

**Isolation of *Bacteroides ureolyticus* from vaginal
discharge of mares**

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Seven *Bacteroides ureolyticus* strains were isolated from the cervix and the clitoral fossa of mares with vaginal discharge. No other bacteria capable of causing metritis or vaginitis were isolated from the samples. The isolated strains could hardly be distinguished from *Taylorella equigenitalis*. Both species were catalase, oxidase and alkaline phosphatase positive, but besides these characteristics *B. ureolyticus* strains produced urease and they did not grow in the presence of 10% O₂. They failed to be agglutinated in a hyperimmune serum raised against *T. equigenitalis*, however *B. ureolyticus* and *T. equigenitalis* were agglutinated in the slide agglutination test in a serum produced against one of our *B. ureolyticus* isolates. Further investigations are needed to clarify the pathogenic role of *B. ureolyticus* in genital infection of mares and other animals.

VIROLOGY AND IMMUNOLOGY

K. NAGY and S. OROSZLÁN

Molecular mechanisms of HIV infection: the role of protease in the early phase of viral replication

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Several aspects of the infection and replication cycle of the human immunodeficiency virus (HIV) are well established. Less known however, are those molecular mechanisms, which after infection precede reverse transcription and lead to proper integration. It has not been established, what triggers initiation of the reverse transcription, and whether this process takes place in the free cytoplasm or in a compartmentalised structure? How could the early replication phase preceding integration be influenced? As a result of our studies, a new model of HIV replication has been suggested, and evidences provided for the active role of retroviral protease (PR) in the early phase of HIV infection. A cellular model allowing study of the single-cycle of HIV replication without the complications of secondary infection was used. Antiviral activity of HIV-1 specific, substrate based, synthetic PR inhibitors (UK 88,947 and Ro 31-8959) in cells infected with the replication competent HIV-1_{IIIB} or the replication defective HIVgpt(HXB-2) was determined. The progression of HIV cDNA synthesis in the cells treated with PR inhibitor was analysed by PCR at 4 h and 18 h post-infection using primer sets recognizing various regions of the viral genome. At 4 h cDNA was detected with all primer pairs used, but after 18 h the *gag* and the *LTR/gag* specific primers were unable to amplify cDNA.

We conclude that PR inhibitors do not affect virus entry or initiation of reverse transcription and that the cDNA transcribed in inhibitor-treated cells is not integration competent, stable cDNA. It is likely that the target of the PR in the early phase of viral replication is the nucleocapsid (NC) protein and its cleavage may be necessary for the formation of a properly assembled cDNA/protein complex (preintegration complex) and/or its transport to the nucleus. These and other results suggest, that PR and NC protein itself and/or its proteolytic fragments may have an active role in the early phase of virus replication.

K. NAGY, V. VÁRKONYI, T. TAKÁCSY, É. BALÁZS, T. TISZA, ZS. SUDÁR
and A. HORVÁTH

**Longitudinal transmission of HIV: the consequence
of viral phenotype**

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Homosexual transmission appears to be predominant mode of transmission of human immunodeficiency virus (HIV) in Hungary. Transmission of HIV is supported by the high infection rate in homosexual partners of HIV infected patients. A total of 229 HIV infected persons were interviewed and tested for the presence of viral antibody. They reported 1334 sexual partners in the past years. Of those 1034 were contacted and also tested for HIV antibody. In average 29% of the sexual partners of HIV positive patients were seropositive in the HIV ab test. Immunological status of 122 homosexual partners of 3 HIV infected male persons had been followed. From the 3 seropositive persons HIV detection and virus isolation were carried out from the peripheral mononuclear cells (PMC). The presence of HIV provirus in the patients' PMCs was detected by PCR using *gag* specific primers amplifying conserved region of genome coding for p24 (CA) protein. HIV was directly isolated from the PMCs of the patients and by cocultivation techniques. Permanent cell lines of lymphoid and monocyte/macrophage origin were productively infected by cell-free virus preparations from the patients. Biological (syntitium induction) and replication characterization of the virus isolates had been determined. Longitudinal investigation among sexual partners of the infected persons revealed that transmission of rapid/high phenotype of HIV isolated resulted more serious consequences regarding induction of AIDS with rapid course and early death than the transmission of slow/low phenotype of HIV. These results suggest the importance of the complex virological analysis of those infected by HIV and the predictive value of these studies.

E. HÜTTER, J. XIE, M. TAKÁCS, A. FALUS and GY. BERENCSI

**Examination of patients suffering from chronic active
hepatitis using hepatitis C virus (HCV)-specific
polymerase chain reaction (PCR)**

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The interferon therapy project launched by the Ministry of Welfare made it possible to begin with the completion of the diagnostics of patients suffering from chronic active hepatitis with the regular application of hepatitis C virus- (HCV) specific polymerase chain reaction. Four pairs of primers have been synthesized on the

basis of (published data, nevertheless, only the pair of primers constructed and pre-tested by A. Falus, using blood samples of Hungarian blood donors, proved to be suitable for the routine-diagnostic tests. More than 500 samples taken from chronic active hepatitis patients, who have been selected for interferon therapy, have been tested. The patients have been preselected according to the international criteria of interferon therapy, therefore the results are not conclusive as far as the general Hungarian population is concerned. About one tenth of the HCV seropositive patients suffering from chronic active hepatitis proved to be negative for HCV RNA (i.e. the concentration of the HCV specific RNA remained below the level of the detection). Each 8th patient selected for interferon therapy on the basis of the international criteria had RNA concentration below the level of detection, using the single pair of primers. These patients were not treated with interferon, since the low level of virus circulation is associated with the ineffectiveness of the therapy according to the published data.

It is an important problem that the international standards for the sensitivity of the test were not available during the trial. Only end point dilution of the samples could be used as the quantitation of the semiquantitative method. The majority of the samples could be diluted 10 to 100 fold, without the loss of positivity. The samples taken before and after the interferon therapy were simultaneously tested. The serum RNA level decreased below the level of detection in the case of about 1 of 5 interferon-treated patients. The densitometric tracing, or visual comparison of the films, however suggested decrease in the concentration of circulating viral RNA in the case of every second patient following treatment. There was a positive correlation between the decrease of RNA level, and the duration of the interferon therapy. Recently the National Institute of Haematology, Blood Transfusion and Immunology reported, that in addition to the 1b sub(genome)type of HCV the sub(genome)type 4 occurs in Hungary at a low frequency. Therefore, it is possible that some of the negative PCR results are fals-positives, since it is not known whether the primers are reactive with sub(genome)types other than 1b. The negative samples are therefore under investigation with IgM-specific HCV test (ABBOTT'), with four pairs of sub(genome)type specific primers, and the earlier introduced primers are tested with serum samples positive for other HCV genome-subtypes. It has to be emphasized, that many fals-positive results were obtained, due to the application of conventional reusable laboratory equipment. The attention of the colleagues has to be drawn to the fact, that PCR can only be done, if all equipments used for the manipulation of sera, and blood samples are disposable.

J. XIE, E. HÜTTER, M. VARGA, K. CSIZMADIA, I. SISKÁ and GY. BERENCSI

**Detection of cytomegalovirus (CMV) DNA sequences
using polymerase chain reaction (PCR)**

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The antigenaemia test is a sensitive method for the detection of reactivated cytomegalovirus infection in immunocompromized patients i.e. transplant recipients. Nevertheless, in some cases, when the granulocyte functions of the patients are

affected, the reactivated viruses will not be taken up by them, and/or the immediate early CMV proteins will not be expressed in them. This difficulty may be overcome by the introduction of the PCR. Since variations of the CMV genomes have been detected by several authors using restriction enzymes, primers, specific for the most conserved (immediate early) DNA region were introduced for the tests (5'-AAGCTT/CCCGCTCCTCCTGAGCACCC-3' and 5'-GAATTC/CCACCCGTGGTGCCAGCTCC-3'), the hexanucleotides at the 5'-ends represent specific palindromes of restriction enzymes, which will allow cloning of the PCR products in the future.

The sensitivity of the test has been measured first. The DNA of 50000 cells used in the antigenaemia test was recovered from the glass slides, and was tested using the standard DNA PCR reaction conditions. Seventeen positive cells could be readily detected under such conditions. The sensitivity of the PCR was shown to be higher than that of the antigenaemia test, since samples containing less than 5 CMV-positive cells per 100000 were found to be positive, too.

Tissue culture fluid containing CMV, Epstein-Barr virus (EBV), varicella-zoster herpesvirus (VZV), human herpesvirus types 1, and 2 (HHSV1, HHSV2) and Marek herpesvirus (MHV) have been tested with the CMV specific pair of primers. The DNA of human herpesvirus type 6 (HHV6) infected cells had to be also tested, since the diagnostic samples from patients might contain latent, or reactivated herpesviruses other than CMV, which could lead to misinterpretation of the results. All tested herpesviruses, even the vaccine strain of Marek disease virus gave positive, nevertheless, distinguishable heterogeneous PCR products with the CMV-specific primers. The comparison of the different products amplified from different herpesviruses led to the suggestion, that in some cases reactivation of HHV6 and not that of the CMV caused the clinical symptoms of the transplant recipients.

E. BARABÁS, K. NAGY and A. HORVÁTH

Significance of prognostical immunologic parameters in asymptomatic HIV infection

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Human immunodeficiency virus (HIV) is known as etiological factor in inducing acquired immunodeficiency syndrome (AIDS). After the primary HIV infection a strong decline in CIM cell count, and in functional activity of lymphocytes can be detected. The CD4 cell count is one of the best prediction factor of the disease outcome. The CD4 count however is limited by variability of biological and individual factors. Moreover the progression and outcome of HIV disease is different in patients with the same CD4 cell count. Thus other prognostical parameters should also be included for follow up. In our study we evaluated the serum level and prognostical role of *beta 2-microglobulin* (B2M) in patients cared in our institute as a part of a complex immunological and virological study in HIV infected persons. Serum antibodies to

HIV-1 was detected by EIA (Organon Teknika). B2M level has been evaluated by competitive fluoroimmunoassay (DELFA, Pharmacia). In addition HBV infection was also determined (HBsAg, Organon Teknika). Sera of a total 700 persons were studied in *cross-sectional evaluation* belonging to high risk group of HIV infection: 149 HIV infected patients, 364 seronegative sexual contacts and 187 persons with STD, venereal diseases and others. B2M level was found elevated (> 3 mg/l) in a significantly higher proportion in the HIV infected group (81/149 or 54%) than in the sexual partners (26/364 or 7%) and in the STD group (8/187 or 4%). HBsAg was detected in 16% of the HIV infected group, $> 1\%$ in contacts and $\sim 4\%$ of the STD group. The prognostical role of B2M was verified by longitudinal studies. The earliest observations were from 1985, the longest period was 7 years (average observation: 4 years). In HIV infected patients monitored from the earliest in this study, serum B2M was higher (4–8.5 mg/l) than in those contracted infection later. Moreover during the observation time in this group 4 out of 6 HIV infected patients have died of AIDS. The outcome of disease was indicated by altering of lymphocyte surface markers monitored during the infection.

Our results further support the findings that the serum level of BIM is an important factor in prediction of AIDS. The actual condition of a larger patient group can be registered by cross-sectional evaluation. Prognosis of the outcome of disease can be made by the longitudinal studies and it helps clinical trials and management of AIDS.

K. SALÁNKI, M. TEPFER and E. BALÁZS

The role of cucumovirus genes in their spread in plant

Agricultural Biotechnology Center, Gödöllő, Hungary and INRA Laboratoire de Biologie Cellulaire, Versailles, France

The type member of the cucumovirus group, cucumber mosaic virus (CMV) is one of the most widespread plant viruses in the world with extremely wide host range. The tomato aspermy virus (TAV) is also member of the cucumovirus group, but its host range is more restricted. For example the CMV is able to infect systemically cucumber, but the TAV does not infect it at all. The cucumoviruses have isometric particles with three functional pieces of positive sense single-stranded RNA and a subgenomic one. The RNA 1, 2 and the RNA 3 can be exchanged between the different cucumoviruses to create pseudorecombinant viruses. Pseudorecombinant viruses revealed that the most important genes for systemic infection localized on the RNA 3, but the exact function of the two genes (3A protein, coat protein) coding by the RNA 3 has not been determined. We wanted to examine the function of these two proteins in the systemic spread of cucumoviruses. We cloned the RNA 3 and determined the complete nucleotide sequences both the P-TAV and R-CMV. We constructed infectious clones of them. Using these infectious clones, we created recombinant viruses to determine the alteration of the symptoms. According to our experiments, the coat protein is the most important factor in the systemic spread of the cucumoviruses.

CS. DRÉN, T. FARKAS and I. NÉMETH

Serological survey on the prevalence of chicken anaemia virus infection in Hungarian chicken flocks

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Chicken anaemia virus (CAV) infection has been demonstrated in Hungarian chicken flocks. The virus has successfully been isolated in cell cultures, and the pathogenicity of the isolated virus strain (CAV-Bia) has been demonstrated by experimental inoculation of susceptible day-old specific-pathogen-free chickens. To test the prevalence of CAV infection in Hungarian chicken flocks, serum samples from meat and layer type parent and broiler chicken flocks were investigated for the presence of anti-CAV antibodies by the indirect immunofluorescence (IF) and virus neutralization tests. A total of 14 parent flocks, between 10 and 62 weeks of age, were tested by the indirect IF test, and all were found to be positive for anti-CAV antibodies. The incidence of seropositivity varied between 40.0 to 93.3%. Out of the 9 broiler flocks, tested between one day and 9 weeks of age, 6 were positive for anti-CAV antibodies, and the incidence of seropositivity was between 10 to 100%. The number of birds tested in a flock varied between 5 to 186. By the virus neutralization test, a total of 10 parent flocks were investigated between 11 to 36 weeks of age, and all were shown to be positive for anti-CAV antibodies. The incidence of seropositivity was between 81.0 to 97.6%. All the 7 broiler flocks, tested between one day and 9 weeks of age, were positive for anti-CAV antibodies, and the incidence of seropositivity varied between 93.3 to 100.0 %. The number of birds tested in a flock was between 30 to 60. These results indicate that CAV is widespread in Hungarian chicken flocks.

A. WITTNER, L. PALKOVICS and E. BALÁZS

Pathogen-derived resistance induced by integrated plum pox virus coat protein gene in *Nicotiana benthamiana*

Institute for Plant Sciences, Agricultural Biotechnology Center, Gödöllő, Hungary

The coat protein (CP) gene from a Hungarian isolate of plum pox virus (PPV-SK68) was engineered into *Agrobacterium tumefaciens* binary vector for expression in *Nicotiana benthamiana* L. plants. The integration of the CP gene was confirmed by PCR technique using genomic DNA preparations. The transcription and expression of the integrated coat protein gene was detected by Northern and Western blots. The resistance was investigated by inoculation of the R1 progeny of the transformed lines with purified PPV-SK68 virion. The different transgenic plants were completely protected or were not protected at all. Six plant lines were tested and two of them were

protected. The resistance of the transformed tobacco lines and the attenuation or absence of the symptom development will be discussed.

GY. VERESS, T. CSIKY-MÉSZÁROS, J. KÓNYA and L. GERGELY

Effects of pregnancy on genital papillomavirus infections

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Different hormonal factors (pregnancy, oral contraception) may exert a significant influence on genital human papillomavirus (HPV) infections, since the presence of progesterone enhances the transcription of the virus. However, epidemiologic data concerning the question are quite inconsistent. For example, some reports confirmed that the detectability of genital HPV infections rises during pregnancy while other studies did not confirm this.

In this study, cytologically and colposcopically healthy pregnant women were subjected to a follow-up examination. Genital samples were collected during early pregnancy (n=50), in the third trimester (n=31) and a few weeks after birth (n=31). Demonstration of HPV DNA from the samples is done by polymerase chain reaction (PCR). Local secretory anti-viral antibodies are detected by ELISA, using synthetic oligopeptides as antigens. We examine the change in the level of antibodies against different viral antigens (E2, E7, L2).

According to our preliminary results, the level of anti-viral antibodies was significantly higher at the time when the third sample was collected (i.e. after birth) than during pregnancy. Results obtained on viral DNA detection will be presented.

G. PREMECZ, A. MARKOVITS, F. NAGY, G. BAGI and I. FÖLDES

Effect of IFN- α on the intracellular calcium concentration of human amnion cells

Microbiological Research Group, B. Johan National Institute of Hygiene, Budapest, National Institute for Alcoholology, Budapest, and National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary

In earlier studies we reported that treatment of human amnion cells (UAC) with natural human IFN- α transiently triggers the breakdown of inositol phospholipids and induces translocation of protein kinase C. On the base of these data it was reasonable to hypothesize that activation of intracellular Ca^{2+} release is also involved in IFN action. Our present results show that addition of IFN- α to cells loaded with Quin 2 (a

fluorescent Ca^{2+} indicator) results in an immediate increase of $[\text{Ca}^{2+}]$; the calcium response to the IFN exposure was dose-dependent and had a markedly biphasic character (i.e. a rapid peak was followed by a pronounced second peak or shoulder). At low concentrations of IFN only the first peak appeared. Interestingly, the calcium response appears to be linked to different biological activity. In cells treated with low doses of IFN the replication of vesicular stomatitis virus RNA was stimulated, while at high concentrations of IFN a significant inhibitory effect was measured. Moreover, a similar biphasic dose-response effect was obtained studying the role of protein kinase C in IFN-action.

M. RUSVAI, B. HARRACH, A. BÁNRÉVI and M. BENKŐ

**Localization and partial sequencing of certain genes
of bovine adenovirus type 2**

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Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest and B. Johan National
Institute of Hygiene, Budapest, Hungary*

DNA fragments of bovine adenovirus type 2 (BAV-2) prototype strain were cloned into pMOB plasmid, and the fragment between *EcoRI* and *SaII* sites (42.5 and 65.2 map units, respectively) was prepared for sequencing. Using *Escherichia coli* DPWC strain, transposons were inserted into the virus specific fragment. Primer sequences complementary to the 5' and 3' ends of the transposon facilitate the sequencing of the viral insert into two directions, left and right to the transposon. Fifty-one different subclones were ranked according to the location of the transposon within the viral DNA insert. The exact place was determined using *SaII* and *BamHI* restriction endonucleases. A set of clones was selected from plasmids containing the transposon with a 300–400 nucleotide difference so it would be possible to carry out the complete sequencing of the estimated 8000 bp-long viral insert after serial sequencing of the clones. DNA was purified using phenol extraction from alkaline minipreps, the sequencing was carried out using Pharmacia A.L.F. sequencer. Using the NCBI BLAST Email server, based on homology to genes from other adenoviruses (human Ad 2, 5, 12, 40, 41 and fowl Ad DNA), genes coded by the obtained DNA were identified as genes of penton protein (pIII), hexon associated protein (pVI) and 100 K protein. Our previous finding was supported: despite of the smaller genom size, the genetic structure of BAV-2 is similar to other adenoviruses.

F. D. TÓTH, P. MOSBORG-PETERSEN, J. KISS, G. ABOAGYE-MATHIESEN,
H. HAGER, C. B. JUHL, L. GERGELY, M. ZDRAVKOVIC, J. ARANYOSI,
L. LAMPÉ and P. EBBESEN

**Studies on interactions between human immunodeficiency
virus type 1 and human cytomegalovirus in coinfecting
syncytiotrophoblasts**

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of Microbiology and Department of Obstetrics and Gynecology, University Medical School,
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Human syncytiotrophoblast (ST) cells produce very low levels of human immunodeficiency virus type 1 (HIV-1), and are non-permissive for human cytomegalovirus (HCMV). When HCMV-infected cells were superinfected with HIV-1, or both viruses were added simultaneously to the cellular targets, a strong, transient stimulation of HIV-1 production was observed. When cells preinfected with HIV-1 were superinfected with HCMV, release of infectious HCMV could be detected. Two-colour immunofluorescence studies have demonstrated that CMV and HIV-1 can coinfect an individual ST cell. Infection of cells with HCMV resulted in the production of moderate levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2). In order to explore the mechanism of enhancement of viral gene expression and replication, double-infected cells were treated with chemically modified monoclonal antibodies (MoAb) to Tat protein of HIV-1, IE1 72-kDa protein of HCMV, GM-CSF and IL-2. These blocking experiments revealed that IE gene products of HCMV transactivate HIV-1 replication by a direct, cytokine-independent mechanism. Conversely, HIV-1 *tat* gene expression plays a major role in the stimulation of HCMV gene expression. Data suggest that HIV-1 and HCMV utilize common cellular transcription factor(s) necessary for a productive viral infection.

T. DALMAY, Z. HAVELDA and J. BURGYÁN

**The mechanism of generation of cymbidium ringspot
tombusvirus defective interfering RNA**

Agricultural Biotechnology Center, Gödöllő, Hungary

Defective interfering (DI) RNAs are deletion mutants of viral genomes. They cannot replicate in the absence of the helper virus since they have lost all essential genes required for viral functions. DI RNAs are heterogeneous in size, ranging from c. 400 to 700 nt. The sequence of the flanking regions of recombination points was analyzed but it was failed to detect any homology or complementarity with each other. It was noticed that a new smaller DI RNA (400 nt) was formed from the known largest DI RNA (DI-13, 700 nt) in planta one month after inoculation and a subsequent

passage. Since the analysis of the flanking sequences did not give any positive result our attention was focused on the deleted sequence itself. Folding this sequence with MFOLD programme, the computer predicted a rather strong base-paired structure. This structure can be found if the whole C block (the last 400 nt) of the DI-13 RNA is folded and the recombination points are located up- and downstream of this 18 nt long stem region. To prove that the locally occurred highly base paired structure in the longer DI RNA is responsible for the generation of the smaller DI RNA the stem was strengthened by in vitro mutagenesis of the full length cDNA clone of DI-13. As it was expected the frequency of recombination was increased when plants were inoculated with the mutant DI RNA. The obtained result suggests that the viral replicase is not always able to unwind this base paired region and copies it, but circumvents it without releasing and produces colinear deletion mutants. This deletion event is not sequence but rather structure specific since it can be reproduced if the appropriate DI RNA sequence is substituted with foreign sequence carrying strong secondary structure. Experiments to decide whether two DI RNA molecules are involved in the recombination events or it is a simply intramolecular deletion are in progress. The results of experiments trying to move the recombination points by creating strong stem structure elsewhere in the deleted sequence, also will be discussed.

AGRICULTURAL AND FOOD MICROBIOLOGY

Z. NAÁR and M. KECSKÉS

The mycoparasitic effect of a *Trichoderma viride* strain in soil affected by vinclozolin

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We studied the influence of vinclozolin on a *Trichoderma viride* strain previously selected for high mycoparasitic activity on sclerotia of *Sclerotinia minor* and fungicide tolerance. A brown forest soil was infected with conidial suspension of *T. viride*, then treated with fungicide suspensions containing 0.01, 0.1, 1, and 10 mg active ingredient per dm³. Seventy sclerotia of *S. minor* per treatment were fixed on glass slides and turned upside down on soil surface. The effect of fungicide on mycoparasitic activity of *T. viride* was recorded as changes in firmness of sclerotia and ability for mycelial germination determined after 4, 8, and 12 days of incubation. We found that (i) the rate of destroyed sclerotia increased with time even in untreated control; (ii) inoculation of soil with *Trichoderma* caused two times higher and significant faster destruction of sclerotia; (iii) the fungicide treatments did not

influence the activity of indigenous antagonists of soil at 0.01 and 0.1 mg per dm³, while higher concentrations (1 and 10 mg per dm³) significantly inhibited it; (iv) the less concentration of vinclozolin increased the destruction of sclerotia in *Trichoderma*-infested soil, but deterred it at higher ones.

J. A. TÓTH, J. KÁTAI and L. SAL

**Effect of herbicides on the growth of *Bacillus subtilis*
and some moulds**

*Ecological Department, Lajos Kossuth University, Debrecen and Department of Soil and Microbiology,
Agricultural University of Debrecen, Hungary*

Applying of chemicals in agriculture results in a modification of agrobiocenosis and their mineral cycles producing unwanted side effects. For understanding the changes, it is important to examine the effect of different herbicides not only on weeds but on the soil microorganisms. To reveal these effects we chose some herbicides namely Patoran 50 WP, Saterb 50 WP, Stomp 330 and Acenit 50 EC to test *Bacillus subtilis* and some fungi. We studied the effect of the chemicals on the germ number in shaken cultures of *B. subtilis*, and on the growth of colonies on solid cultures at moulds in laboratory. The tested fungi were *Aspergillus niger*, *Fusarium culmorum* and *Trichoderma viride*. *B. subtilis* was cultivated on meat peptone medium, the fungi were tested on Czapek-Dox agar. The concentrations of herbicides applied in cultures were 5-, 10-, and 100-fold dilution of the saturated aqueous solution. Ten-fold and 100-fold dilutions of Patoran 50 WP containing metobromuron stimulated the growth of *B. subtilis* while the five-fold dilution had already slight inhibitory effect. The stimulating effect may be the result of a process in which *B. subtilis* metabolized Patoran. In contrast with *B. subtilis*, the growth of moulds was inhibited by Patoran WP as the concentrations were raised. Saterb 50 WP with terbutin agent inhibited the growth of *B. subtilis* and the moulds. With increasing the doses, the inhibition increased. Stomp 330 with pentimetalin agent had an inhibitory effect of practically 100 % on the growth of *B. subtilis*. Similarly strong inhibitory effect was found at moulds. Acenit 50 EC with acetochlor also inhibited the growth of *B. subtilis* and that of the moulds, too. This chemical was the most toxic on moulds.

J. A. TÓTH, J. KÁTAI and L. SAL

**Effect of PAH compounds on the growth
of *Bacillus subtilis* and some moulds**

*Ecological Department, Lajos Kossuth University, Debrecen and Department of Soil and Microbiology,
Agricultural University of Debrecen, Hungary*

Polycyclic aromatic hydrocarbons (PAH compounds) with toxic effect, such as Anthracene and Phenanthrene can reach the soil and living waters by industrial sewage, sewage mud, raw oil, etc. They can cause even the death of living organisms there. Anthracene and Phenanthrene being hardly resistant compounds can accumulate in the mud of living waters. Among fungi, there are organisms with capacity of decomposition of Anthracene and Phenanthrene like among bacteria. Kosinkiewicz et al. prepared an *Azotobacter* species from soil that can decompose Anthracene. *Pseudomonas fluorescens*, *Pseudomonas paucimobilis*, *Alcaligenes denitrificans* (Weissenfels et al.) and *Arthrobacter polychromogenes* (Heitkamp et al.) have the capacity of the decomposition of Phenanthrene. Among fungi, *Trichoderma hartianum* (Ermish et al.) can decompose Anthracene. *Aspergillus ochraceus*, *Cunninghamella elegans*, *Penerochaete chrysosporium*, *Saccharomyces cerevisiae* and *Syncephalastrum racemosum* can metabolize both Anthracene and Phenanthrene (Sutherland).

In our study we try to reveal the effect of Anthracene and Phenanthrene on some test micro-organisms in laboratory. How they influence the number of *Bacillus subtilis* in shaken culture, and the growth of colony diameter of *Aspergillus niger*, *Fusarium culmorum* and *Trichoderma viride*. The culture medium was meat peptone in the case of *B. subtilis* and that of Czapek-Dox agar for moulds. The concentration of Anthracene and Phenanthrene in medium cultures was 5, 10 and 100-fold dilution of the saturated solution. Anthracene and Phenanthrene strongly inhibited the growth of *B. subtilis*. Decrease in cell count was almost 100% even at the lowest concentration. At higher concentrations inhibitory effects were stronger. At moulds Anthracene and Phenanthrene only slightly inhibited the colony growth comparing to the control. Increasing the concentrations the toxic effect only slightly increased. There were no significant differences between the effect of the two chemicals. In contrast, Anthracene and especially Phenanthrene proved to be strong inhibitors on *B. subtilis* cultures. Consequently the moulds tested are more tolerant against these chemicals than *B. subtilis*.

J. KÁTAI, B. HELMECZI and M. BESSENYEI

**Correlation among the physical, chemical characteristics
and microbiological activity of some soil types**

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In this study, 16 different soil samples belonging to 14 types were selected for comparative analyses. We clustered these soil samples on the basis of their physical, chemical and microbiological characteristics and site value. We looked for relationships among the mentioned parameters with correlation survey. On the basis of our results the clay and silt content, soil plasticity according to Arany and the hygroscopic capacity of the soil types were the most essential among the investigated physical characteristics. Among the chemical properties, pH (and the related features) and the organic content of the samples were the most significant in the cluster analyses. The microbiological parameters were equally important in the classification of the soil types. The main types of the meadow and chernozem soil samples and those soil samples which had little microbiological activities were separated definitely from each other in the cluster analyses. When the cluster analyses were performed on the basis of the site values of the soil samples, the meadow and chernozem soil types and those with low productive capacities fell into separated groups too. The correlation studies showed relationship between the soil microbiological characteristics and physical-chemical ones in the case of some parameters. We give account of some relationships among soil microbiological characteristics. The site values showed significant correlation with the cellulolytic activity and the number of nitrifying bacteria. Thus it appears that the physical, chemical and microbiological characteristics of the different soil types have to be considered in a complex manner in order to get applicable information on their productive capacity.

B. HELMECZI, J. KÁTAI and M. BESSENYEI

The effect of chrome on the growth of some fungi

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The sewage water of Debrecen is partly industrial and partly communal in origin (40 and 60%, respectively). The sewage water was concentrated by 4–6% dry matter content during the process of gravimetric concentration and the sewage-sludge has been placed to agricultural areas since 1983 (on approval of the Ministry of Agriculture and Food Industry, Ministry of Health and National Water Conservancy Office). Since 1984 the sewage-sludge injected in the quantity of 9.6 t/ha/year, into the depth of 42 cm of humous sand and meadow chernozem soil type at the area of Kossuth Farm of Debrecen. The sewage-sludge contains lot of heavy metals,

particularly chrome (700 mg/hg). It can originate from the local leather factory. At the same time with the starting of injection technology an experiment was put into on the area. The aim of it was to state the effect of sewage-sludge on the soil and plants too. Parallel with the experiment we carried out soil microbiological investigations in 1990–91. The results were summarized in 1993. The number and activity of bacteria belonging to different physiological groups increased on the treated plots while the number of the microscopic fungi usually slightly changed but it definitely decreased in meadow chernozem soil type. After that we investigated the effects of chrome on microscopic fungi occurring in soil, in clear culture on peptone–glucose agar medium. The trials were carried out by MILLER's agar-block method (1979), the agents were calculated to 2–160 mg/kg Cr_2O_3 , K_2CrO_4 , $\text{K}_2\text{Cr}_2\text{O}_7$. The growth of fungi was stimulated by smaller doses of chrome (2–40 mg/kg) in some cases while it was inhibited and destroyed by larger doses (80–160 mg/kg).

J. BECZNER, J. TELEGDI and E. KÁLMÁN

**Effect of biocides on the corrosion activity
of *Desulfovibrio desulfuricans***

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Biodeterioration of materials – partly desirable (biodegradation of wastes), partly harmful (i.e. biocorrosion of metals, biodegradation of paper, leather, wood etc.) – frequently occurs under natural conditions. The formation of biofilm on the surfaces is of primary importance in the biodeterioration and biocorrosion. Antimicrobial activity of different biocides were investigated in vitro against anaerobic sulphate reducing *Desulfovibrio desulfuricans* (strain ATCC 7757). For longer storage the strain was lyophilized. The subcultures were maintained in the medium recommended by ATCC (ATCC Medium 1249), under nitrogen. Medium without added sulphide was used for cell counting. The incubation temperature was 37 °C, the cultures were generally incubated maximum for 7 days. Different biocides were investigated for their antimicrobial efficiency as a function of concentration, using glutaraldehyde control. Glutaraldehyde was very effective, even in small concentration. Out of the biocides tested AS I, AS II, M I and M II inhibited the growth of *D. desulfuricans* to different degree. The effect of biocides on the formation of biofilm on mild steel coupons was investigated in the presence and absence of detergent Tween 80 (1%). After 4 days of incubation growth of bacteria was detected in all cases except for GA, 100 ppm, with or without detergent. AS II was equally efficient. Effect of biocide was tested against the existing biofilm. Neither of the biocides in the concentration investigated was practically effective against the bacteria in the biofilm, as it was shown by the cell count numbers. Electrodes incubated in ATCC medium 1249 with or without *D. desulfuricans* for 25 days at room temperature were investigated. The biofilm formation was extensive, and underneath corrosion of the metal occurred; the presence

of bacteria was detected using epifluorescence microscopy. It is clear that the biocides are effective against the planktonic *D. desulfuricans* cells, and to a smaller extent, if at all, against the sessile cells in the biofilm, at least in the concentrations tested. It is interesting that the biofilm formation was not inhibited, in the presence of detergent, except for the glutaraldehyde. The bacteria in the biofilm are well protected, even against the effective glutaraldehyde.

H.E.A.F BAYOUMI, B. BÍRÓ and M. KECSKÉS

In vitro metal tolerance of some symbiotic *Rhizobium leguminosarum* and *R. loti* strains

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Total number of biological N₂-fixers, their effectivity and/or N₂-fixing capacity might change after a long-term deposition of liquid manures and sewage sludges, containing heavy metals (Martensson and Witter 1990, Giller et al. 1989). In vitro selection of tolerant bacteria with good performance and inoculation with them, might help for reinedlate of these heavy-metal contaminated arable fields. The effect of different microelements (Al³⁺, Cd²⁺, Co²⁺, Cu²⁺, Fe²⁺, Mn²⁺, Mo²⁺, Se²⁺ and Zn²⁺) mainly as sulphate and chloride salts were examined (in 25, 50, 100, 200 and 400 µM concentrations) on the growth of four *Rhizobium leguminosarum* bv. *viciae* and one *R. loti* strains. Optical density (OD 550 nm) of inoculated tubes were tested in liquid yeast-mannitol media (YEM, Vincent 1970, modified by one of us (Hamuda Bayoumi, 1992), after 48 h incubation, in rotary shaker at 28 °C. Data of triplicate tubes are shown in per cent of control (inoculated, metal-free) samples. Among the metals examined, Co²⁺ and Cu²⁺ proved to be the most toxic (more than 50% reduction of the cell number). Se²⁺ and Fe²⁺ on the other hand were stimulative and essential for the growth. Salts of Mn²⁺, Mo²⁺ and Al²⁺ did not cause any significant difference in the above-mentioned media. Sulphate form of different metals however, was more toxic, than the chloride form. Sensitivity against the microelements, examined depended mostly on the strains and not on the species. There was a high diversity among the *R. leguminosarum* bv. *viciae* isolates. An especially tolerant strain "Lóbab Z" proved to be a successful candidate for *Vicia faba* L. inoculation in soils, affected by heavy-metals.

N. KUCSMA, H.E.A.F. BAYOUMI, GY. VÁRADY, B. BÍRÓ and M. KECSKÉS

**Effect of metal-combinations on some strains of various
Rhizobium strains**

Department of Microbiology, University of Agricultural Sciences, Gödöllő and Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences, Budapest, Hungary

Tolerance of different strains of *Rhizobium* species (*R. leguminosarum* bv. *viceae*, bv. *phaseoli*, bv. *trifolii*; *R. loti* and *R. meliloti*) were examined at 10, 20, 40, 80 and 160 μM metal ions (Al^{3+} , Cd^{2+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Mn^{2+} , Mo^{2+} and Zn^{2+}), mainly as the form of sulphate and chloridic salts in liquid yeast-mannitol media (YEM – Vincent 1970, modified by us – Hamuda Bayoumi, 1992). Effect of 10 or 20 μM Fe^{2+} combined with the above-mentioned metals were also tested after 48 h incubation, in rotary shaker at 28 °C. Growth rate (optical density, OD 550 nm) of different strains were checked. Data of triplicate tubes are shown in per cent of control (inoculated, metal-free) samples.

Among the metals examined, Co^{2+} , Cu^{2+} , Cd^{2+} and Al^{2+} proved to be the most toxic and extension of sensitivity were decreasing also in this order. Mn^{2+} and Mo^{2+} on the other hand proved to be least toxic or stimulative in small concentrations. 20 μM Fe^{2+} in combination with any amounts of the microelements (mentioned above) reduced the growth rate of all the strains. 10 μM Fe^{2+} , however compensated the harmful effects of different metals, so application of Mn^{2+} (10–80 μM) and Fe^{2+} (10 μM) were especially stimulative on the growth rate of all the strains.

Among the strains of various *Rhizobium* species there were no significant differences in tolerance, *R. leguminosarum* bv. *viceae* however had a particularly outstanding diversity. After the results of in vitro tolerance, strain "Lóbab Z" might be a successful candidate for *Vicia faba* inoculations on the arable fields affected by heavy-metals.

M. JUHÁSZ-ROMÁN

**Examination of lactic acid bacteria in alcoholic
and alcohol-free beverages**

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Lactobacillus brevis seems to be the most frequent species in spoilage of beer, fruit juices and wines. The thermal resistance factor for this species was different, depending upon the composition of beverages. Pasteurization was the most effective at high alcohol content and at low pH. Only one process, malolactic fermentation by *Leuconostoc oenos* might be useful, especially for wine making. This species of lactic acid bacteria is widely popular in Western Europe, as starter for induction of the biological deacidification of the hard acid red wines. A lot of strains of *L. oenos* were

collected from the historical winemaking regions of Hungary. In the industrial practice it is a problem to make a vigorous inoculum in suitable quantities. Fructose and tomato juice extract had stimulatory effects for the growth of *L. oenos*.

I. NAGY, S. VERHEYEN, A. DE SCHRIJVER, G. SCHOOF, J. VANDERLEYDEN
and R. DE MOT

**Biodegradation of thiocarbamate herbicides
by *Rhodococcus* sp. NI86/21**

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Rhodococcus sp. NI86/21 can act as a biosafener, protecting inoculated maize plants against the phytotoxic effects of the thiocarbamates EPTC, butylate, and vernolate by degradation of these herbicides. By two-dimensional electrophoresis, two major and two minor proteins induced in cells grown in thiocarbamate-containing medium were identified. These proteins were isolated by preparative two-dimensional electrophoresis, and their *N*-terminal amino acid sequence determined. By oligo-hybridization, the corresponding DNA fragments were identified and sequenced. One of the proteins induced at high level was a NAD-dependent dehydrogenase active on aliphatic aldehydes. The second abundant protein was identified as a novel type of cytochrome P-450 enzyme. Downstream of the latter gene, a [2Fe-2S] ferredoxin and a ferredoxin reductase gene were located. A putative regulatory gene encoding a new member of the AraC/XylS family of positive transcriptional regulators was divergently transcribed from the cytochrome P-450 gene. Evidence for the involvement of this cytochrome P-450 system in EPTC degradation was obtained by complementation experiments using EPTC-minus *Rhodococcus* strains. GC-MS analysis demonstrated the production of *N*-dealkylated EPTC during *Rhodococcus* sp. NI86/21 EPTC degradation. Cytochrome P-450 mediated *N*-dealkylation and conversion of the released aldehyde by aldehyde dehydrogenase initiate EPTC catabolism in *Rhodococcus* sp. NI86/21. Two minor proteins were identified as a chloroperoxidase and a *N,N'*-dimethyl-4-nitrososaniline-dependent oxidoreductase. The potential involvement of these enzymes in thiocarbamate degradation is currently further investigated.

CS. DOBOLYI, I. DAMJANOVA and K. RABÓCZKI

Spore types of AM inhabiting in marshlands in Hungary

Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary

Spore types of arbuscular mycorrhizas (AM) were isolated from soil of four marshland biotops. Using the wet sieving method we could obtain AM spores of different quantity from each sample of investigated soils. The rhizosphere of dominant marshland plants contained 10^2 – 10^3 spores per 100 g soil, the most (1800) was in that of the Sárrét area, the less (200) was in the Szilas area. Seasonality was characteristic with an optimum in March. Twelve spore types belonging the genera *Glomus*, *Scutellospora*, *Sclerocystis* could be differentiated. The incidence of different spore types depended on biotops and time of sampling. Two spore types (type SL-1 and type SL-3) appeared in each biotop, their incidence did not exceed 11%, while the other ten spore types could be found only in a single biotop. In each tested soil a dominant spore type was identified with an incidence exceeding 50% (85% of type SL-9 in the Sárrét area, 54% of type SL-12 in the Szilas area and 52% of type SL-5 in the Dombóvár area). AM colonization in roots of the characteristic plant species was also followed with the trypan blue staining method.

J. KUKOLYA and CS. DOBOLYI

Comparative study of proteolytic microorganisms inhabiting soil and compost

Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary

The microorganisms having outstanding proteolytic activities are bacteria and generally belong to the genus *Bacillus*. We isolated microorganisms of proteolytic activity with gelatin-containing agar plate. After a preliminary testing we selected 5 mesophilic actinomycetes, 8 fungal strains, 7 mesophilic and 5 thermophilic bacteria for further investigations. For a comparative study the pH, the total germ count and the esterase, keratinase and the protease activity of fermentation broth were tested; esterase activity with the measurement of the hydrolysis of FDA, that of proteinase with modified Kunitz-test were estimated. A mesophilic *Bacillus* strain(C48) proved to be most active for protease, while among the tested fungi a *Malbranchea sulphurea* strain (GB10) showed the highest esterase and protease activity. In a detailed examination of the protease of the strain *Bacillus* sp. (C48) proteinase K-equality, pH-optimum and thermal stability of the fermentation broth were tested. On the basis of the results (the activity of the partially purified enzyme showed an optimum value at pH 10.0, a half life was 30 min at 70 °C and was totally inhibited by PMSF, while not by EDTA), this enzyme can be classified as serine protease. We achieved the long-term preservation of

the selected strains with freeze drying and L-drying. The viability of lyophilized microorganisms were studied during storage for 6 months. The mean logarithmic value of the viable count of the bacteria decreased only one unit, and the protease activity remained at high level. The preservation caused large decrease on the viable cell count of the streptomycetes and the fungal strains, but only the *Streptomyces*. sp. (SB4) protease activity decreased after the maintenance.

I. KISS and J. BECZNER

Effect of combined treatments on shelf-life of sliced pork meat

Department of Refrigeration and Livestock Products Technology, University of Horticulture and Food Industry, Budapest and Department of Microbiology, Central Food Research Institute, Budapest, Hungary

Sliced deboned pork meat (*M. longissimus dorsi*) was packed in polyethylene-polyamide-polyethylene foil under vacuum or in CO₂ atmosphere. The irradiation was carried out with a ⁶⁰Co gamma source in 0–4 kGy dose range. The samples were stored at 3–5 °C. The cell count of samples was studied as a function of packaging, irradiation dose and storage time. The survivors were determined with MPN method in nutrient broth after incubation at 30 °C for 72 h, and at 13–15 °C for 7 days. The number of lactobacilli were determined on Rogosa agar with pour plate technique at 37 °C. Ionizing radiation (2 kGy) increased the shelf-life at 3–5 °C temperature over 28–35 days in case of vacuum packaging, whereas that of the unirradiated sample was 6–8 days under the same conditions. Combination of irradiation (2 kGy) and CO₂ atmosphere increased the storability up to 25–30 days. The psychrotrophic bacteria, the dominant spoilage microflora of the meat, were sensitive to irradiation. Due to this relatively high radiosensitivity the number of psychrotrophs was reduced by 2 log cycles with 2 kGy radiation dose in vacuum- and CO₂-packed samples. During storage the growth of *Lactobacillus* sp. was practically not inhibited by vacuum- or CO₂-packaging. The irradiation (2 kGy) reduced the viable cell count and inhibited the growth rate of the surviving bacteria and therefore influenced the shelf-life.

I. VIDÁCS and J. BECZNER

**Effect of combined treatments on the survival of
spore-forming bacteria**

*University of Horticulture and Food Industry, Budapest and Department of Microbiology, Central Food
Research Institute, Budapest, Hungary*

The survival of *Bacillus cereus* was studied in combination of heat treatment (100 °C, 0–20 min), irradiation (0–6 kGy), pH (5–7) and salt content (0–5.5%) of the growth medium. The treatments were carded out in 10 cm³ phosphate-buffer with 10⁶ spores/cm³ spore suspension. The number of residual viable spores was determined with pour plate technique on TGE agar. Incubation lasted for 3–5 days at 37 °C. The most efficient single treatment in the intervals studied was heat treatment, which combined with irradiation showed a very significant reduction in the viable cell count. A change in pH and salt content influenced the cell count as well, but the effect of these in the intervals studied did not reach the effect of the heat treatment combined with irradiation.

O. REICHART

**Effect of the pH and potassium-sorbate concentration
on the growth of *Zygosaccharomyces bailii***

*Department of Microbiology and Biotechnology, University of Horticulture and Food Industry,
Budapest, Hungary*

The growth of a *Zygosaccharomyces bailii* strain with extreme high sorbate resistance, isolated from spoiled soft drink was studied in synthetic broth as a function of the pH and K-sorbate content of the medium. The initial pH of the Tryptone-Glucose-Yeast extract (TGY) media were 2, 3, 4, 5 and 6, and the K-sorbate concentrations at every pH's were 0, 0.5, 1.0, 1.5 and 2.0 g/dm³. As an inoculum 5% (v/v) suspension of *Z. bailii* grown on sorbate-free TGY broth was used. The growth experiments were performed in test tubes at 30 °C and the growth curves were measured spectrophotometrically at 550 nm. Evaluation of the growth curves indicated that decreasing the pH and increasing K-sorbate content (increasing the concentration of the undissociated sorbic acid) resulted in a longer lag-period and decreased the growth rate and maximum cell-concentration. Even in the case of the maximum sorbic acid concentration (pH=2, K-sorbate=2.0 g/dm³) a significant growth was measured. Mathematical models describing the effect of the pH, sorbic acid and sorbate concentration on the growth rate and lag phase were constructed and comparatively evaluated.

J. FARKAS and É. ANDRÁSSY

Behaviour of *Listeria* in an extended shelf-life chilled meat product

Department of Refrigeration and Livestock Products Technology, University of Horticulture and Food Industry, Budapest, Hungary

Our previous experiments demonstrated that shelf-life of a refrigerated, vacuum-packaged, ready-to-fly, minced meat product, 'tenderloin rolls' can be considerably extended by combinations of slight reductions of its pH and water activity and a gamma radiation dose of 2 kGy. In order to assess microbiological safety of such extended shelf-life chilled product, in the present studies *Listeria monocytogenes* was inoculated into the samples before radiation treatment at approx. $10^3/g$ and $10^4/g$ inoculum levels. The radiation treatment resulted in approx. 2-log cycles reduction of the colony forming units of *L. monocytogenes*, somewhat less in that of lactic acid bacteria, while one log cycle decrease was observed in the total aerobic plate count. *Enterobacteriaceae* were essentially eliminated by the radiation treatment. *L. monocytogenes* failed to grow in the unirradiated product held at +2 °C for 4 weeks plus one week at 10 °C, however, it survived well, while residual populations of *L. monocytogenes* decreased gradually in the irradiated product during the same post-irradiation storage. For the fourth week of refrigerated storage, lactic acid bacteria became the dominant component of the microflora in all experimental batches. Our data suggest that combination treatment as applied not only extends the shelf-life of this chilled product but it may have a role in controlling psychrotrophic pathogenic bacteria such as *L. monocytogenes*.

R. KISS and M. RODLER

***Listeria monocytogenes* strains of food origin in Hungary**

National Institute of Food Hygiene and Nutrition, Budapest, Hungary

Food and environmental samples were taken in food stores, buffets, salad bars and various catering institutions. Twenty-five gram samples were examined in 225 ml brain-heart broth made selective with CNA. After 2–4 days incubation at 30 °C, subculture were made on Columbia agar with 5 % beef blood incubated at 30 °C in Generbox in microaerob atmosphere. Identification was performed by catalase and CAMP tests, beta hemolysis, motility at 20 °C. The following tests were used: rapid API 20 Strep, rapid ID 32 Strep, API *Listeria* (bio Mérieux). The rapid strips were incubated for 4 h at 37 °C.

Distribution of the positive samples: 27 brawn, 7 deep-frozen meatloaf, 13 buffet meals and salads, 18 cakes with egg, cream, honey, 2 sheep cottage cheese, 8 cheese, 8 sausages, ham, 11 minced meat, 1 raw milk, 2 raw chicken, 7 ice-cream, 2

environmental cleanliness sample. Out of 1344 samples 107 were positive (7.9%). The serotypes were: 99 – St 1/2a; 1 – St 1/2b; 7 – St 4b.

D. BÁNÁTI and H. STEGEMAN

**The effect of antimicrobial treatments on the lag phase
of *Yersinia enterocolitica***

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Industry, Budapest, Hungary and RIKILT-DLO, State Institute for Quality Control of Agricultural
Products, Wageningen, The Netherlands*

The number of cases caused by food-borne diseases are increasing continuously world-wide. The micro-ecological factors should be considered seriously in food processing because of their determining role in the growth of pathogens and other bacteria in any food system. We carried out model experiments to study the microbiological safety of some chilled foods inoculated with psychotrophic pathogens such as *Yersinia enterocolitica*. The growth of this bacteria was detected by an impedimetric method with Matthus instrument at an incubation temperature of 25 and 10 °C – close to the optimal and at suboptimal temperature representing cold storage. Some characteristics of the growth kinetics were analysed such as generation time and lag phase which could be calculated based on the change of detection time of treated and untreated populations. The antimicrobial treatments were: heat treatment, irradiation and frozen storage. The last two were combined and applied also in a reverse order. The lag phase of *Y. enterocolitica* treated by any of these factors was always longer than that of the untreated one. Heat treatment had the strongest effect on this part of the growth phase. After a certain reduction in number of bacteria the heat treated population had longer lag phase than the irradiated one so the former one had a more damaging effect to the bacteria. Suboptimal temperature had great influence on the increase of the lag phase. The D_{10} value of the examined population was 47 Gy and 87 Gy when irradiated in frozen state. The sequence of these treatments had an additional effect on the (increase of the) lag phase of *Y. enterocolitica*. When irradiation was applied after frozen storage the number of survivors was higher than in case of irradiation followed by frozen storage.

R. KISS, G. SZITA and G. TÓTH

**Detection of enteropathogenic *Escherichia coli* strains
from food**

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Budapest, Hungary*

This work was performed by the use of Fluorocult LMX Broth, X-Broth (bio Mérieux) and Fluorocult *E. coli* O157:H7 Agar (Reanal, Budapest) which indicates the MUG positivity of the strains and because of the sorbite component of the agar, helps in the isolation. The aim of the present examination was to check in model experiments whether the Fluorocult LMX Broth, X-Broth and Fluorocult *E. coli* O157:H7 Agar are suitable for the detection of different pathogenic *Escherichia coli* strains. Later the method was introduced in our routine food control work. Dilution series were made from the strains occurring most frequently in the course of enteric diseases in Hungary. These are O26:K60; O55:K59; O111:K58; O128:K67:H12; O124:K72 and O157:H7. We found that all the three media were useful for the detection of *E. coli*. In routine examinations we found 97 *E. coli* strains.

CS. MOHÁCSI-FARKAS, J. FARKAS and A. SIMON

**Thermal denaturation of bacterial cells examined by
differential scanning calorimetry**

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Differential scanning calorimetry (DSC) with a SETARAM microcalorimeter was used to detect thermal transitions in cells of *Listeria monocytogenes*, *Escherichia coli* and *Lactobacillus plantarum*, suspended in pH 6.8 physiological phosphate buffer. DSC-scanning at 0.5 °C/min showed multi-peaked thermograms. Thermograms taken from 10 to 99 °C temperature of growth and a series of endothermic transitions began to appear between 50 to 60 °C, in the temperature range, where the heat destruction of vegetative cells occurred. Our observations strengthen the premise that the process responsible for thermal death is an irreversible thermal denaturation of a crucial proteinaceous component of the cells and it is strongly influenced by the pH of the suspending citric/phosphate buffer. Heat denaturation/melting of DNA required a higher temperature than cell killing, and did not correlate with thermal stability of the bacteria. Heat denaturation of the DNA in whole cells was more or less reversible when heated at neutral pH. However, it became irreversible when they were heated in acidic buffer.

R. KISS and E. VÉGH

**Examination of foods with increased histamine contents –
microbiological characteristics**

National Institute of Food Hygiene and Nutrition, Budapest, Hungary

As bacteria present in foods, due to decomposing proteins and amino acids, affect the histamine contents of foods, examinations were performed in connection with the microbiological of histamine containing foods. Our attention was called to the examinations of anchovy-ring, herring and "brindza" (cheese of sheep) by literary data. A total of 42 examinations were performed. Histamine detection was performed by use of fluorometric determination after extraction. Histamine values of 200 mg/kg (in the case of anchovy-rings, 500 mg/kg) are rejected at present in Hungary, taking into consideration the international data. The identified bacteria were: anchovy-ring: *Clostridium perfringens*, *Propionibacterium acnes*, *Bacillus cereus*, *Enterococcus faecium*, *Staphylococcus aureus*; herring: *Actinomyces israelii*, *B. cereus*, *Staphylococcus epidermidis*, *Listeria grayi*, *Serratia* sp.; brindza: *S. aureus*, *Escherichia coli*, *Klebsiella oxytoca*, *Hafnia alvei*.

Our samples met in every case, from microbiological aspects, the presently valid degree in Hungary, but were rejected, on the basis of histamine values. In the course of our examinations histamine values between 58.5 mg/kg and 1000 mg/kg were measured.

MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

A. MARÁZ, A. GELETA, I. MAGYAR and G. BUJDOSÓ

**Anaerobic metabolism of L-malic acid by
*Schizosaccharomyces pombe***

*Department of Microbiology and Biotechnology and Department of Enology, University of Horticulture
and Food Industry, Budapest, Hungary*

Decomposition of L-malic acid by yeasts under anaerobic conditions is prerequisite by the presence of the cytoplasmic malic enzyme (E.C.1.1.1.38) but the rate or its metabolism is influenced by other metabolic processes (e.g. transport and ethanol productivity), too. In that case when malic acid fermenting yeast strains are intended to be applied for enological purposes, determination of the main physiological

factors influencing malate metabolism and that of the production of fermentation byproducts (e.g. flavours, aromas, H₂S) is necessary. As the result of our screening procedure, *Schizosaccharomyces pombe* strains were selected showing one or more of the following physiological characteristics: good growth rate under acidic conditions (pH 3.5) in anaerobiosis; good L-malic acid fermentation rate; tolerance for 10% ethanol; weak H₂S production. From the results of determination of growth and malic acid decomposition kinetics it was concluded that the rate limiting factors are the low pH and the ethanol tolerance. Strain improvement program was started when selected strains were hybridized by sexual and somatic hybridization.

ZS. HAMARI, B. TÓTH, J. VARGA and F. KEVEI

**Transmission and recombination of mitochondria
among black *Aspergilli***

Department of Microbiology, Attila József University, Szeged, Hungary

The individual isolates of species belonging among black *Aspergilli* (Section *Nigri*) exhibit a wide range of genetic diversity. *A. niger* is a species aggregate in which large numbers of collection strains and field isolates have been examined for various molecular characters. A high degree of polymorphism was observed in the mitochondrial DNA (mtDNA) and nuclear ribosomal RNA genes (rDNA) of these strains. Several groups can be distinguished on the basis of studies. Considering the *Sma*I-generated restriction patterns of rDNA and the *Hae*III-*Bgl*II digested mtDNA profiles, these strains can be divided into three main groups (rDNA groups I, II, and III corresponding to mtDNA groups 1, 2, and 3, respectively; 4). MtDNA transmission between strains representing different RFLP groups was carried out by using a mitochondrial oligomycin resistant mutant (N909, constructed by Alex Coenen), which exhibits type Ia mtDNA, and type I rDNA patterns. The oligomycin resistance marker was successfully transferred by protoplast fusion into isolates carrying type Ia, Ib, 2a, 2b or 3 mtDNAs. MtDNAs were transmitted even between strains showing full incompatibility in respect of nuclear complementation. The transfer resulted in recombination of the mitochondrial genomes of the donor and recipient strains in most cases, except in the case of Ia-Ia transfer. Recombination readily occurred even between mtDNAs of strains representing different species like closely related *A. niger* and *A. tubingensis*, but we could not transfer the oligomycin resistance marker to more distantly related black *Aspergilli* (*A. carbonarius*, *A. japonicus*). Interestingly, different types of recombinant mtDNAs were observed in the progeny of a single oligomycin transmission experiment (e.g. the *Hae*III-*Bgl*II digested mtDNA patterns of the recombinants obtained from the Ia-2b and Ia-3 transfer. Study of the nature of recombinant mtDNAs is in progress.

J. VARGA and E. RINYU

**RFLP analysis of species of section *Fumigati*,
genus *Aspergillus***

Department of Microbiology, Attila József University, Szeged, Hungary

Isolates and collection strains of *Aspergillus fumigatus*, and representatives of other related species belonging to section *Fumigati* of the genus *Aspergillus* were compared for their micromorphological features, mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) patterns obtained by using restriction enzymes. The ascospore ornamentation of the sexual species examined by scanning electron microscopy was found to be species-specific. The *HaeIII*-generated mtDNA patterns, and *SmaI*-digested repetitive DNA patterns of all except one *A. fumigatus* strain were the same. The mtDNA patterns, and the *SmaI*-generated repetitive DNA profiles were characteristic to the other species examined, except for the two *A. viridinutans* strains, which displayed intraspecific variability. Hybridization of the *A. nidulans* ribosomal repeat unit to the *SmaI*-digested DNA patterns proved that most of these repetitive bands represent parts of the ribosomal RNA repeat unit, but revealed the presence of non-homologous repetitive bands which probably correspond to parts of the intergenic spacer region of these strains. The dendrogram produced by the unweighed pair group method from the mtDNA and rDNA RFLP data indicates a possible close relationship of *A. citrisporus*, *A. auratus* (= *Neosartorya aurata*) and *A. paleaceus* (= *N. straminea*), and of *A. fumigatus* and *A. fischerianus* (= *N. fischeri*), respectively. Strain FRR 1266 possibly represents a new asexual species of section *Fumigati*, since it showed unique mtDNA, rDNA and isoenzyme profiles; classical taxonomic reexamination of this strain is in progress.

CS. VÁGVÖLGYI, T. PAPP and L. FERENCZY

**Intraspecific variation in isozyme patterns
of *Mucor piriformis* isolates**

Department of Microbiology, Attila József University, Szeged, Hungary

Mucor piriformis has been reported to be an important postharvest pathogen of numerous fruits and vegetables. In our work isoenzyme analysis was used to investigate the intraspecific variability in *M. piriformis*. Eight enzyme activities, acid phosphatase (ACP), alcohol dehydrogenase (ADH), catalase (CAT), alfa-esterase (EST), glucose-6-phosphate dehydrogenase (G6P), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and superoxide dismutase (SOD) have been tested for 10 (+), 9 (-) and 10 neutral isolates. The variability of isoenzyme patterns have been found to be lower than expected. ADH, MDH and EST patterns could clearly

differentiate the strains with mating abilities from neutral ones. All plus and minus strains revealed the same pattern in each case. Enzyme profiles for neutral strains were also nearly invariable, but different from those of the mating strains. ACP, CAT, LDH and SOD activities revealed the same patterns for all the 29 isolates. G6P enzyme profiles were found to be more variable with no correlation with mating abilities.

R. NAGY and L. HORNOK

Mini-chromosomes of *Fusarium sporotrichioides*

Agricultural Biotechnology Center, Gödöllő and Department of Microbiology, Agricultural University, Gödöllő, Hungary

Karyotype variability of 26 *Fusarium sporotrichioides* isolates from different plant and geographic origins were restricted to mini-chromosomes smaller than 2.0 Mb. In order to investigate the origin of these interesting structures pooled DNA-fragments of the 1.43 and 1.17 Mb mini-chromosomes of two typical strains were hybridized to the whole chromosomal sets of all strains. The strong hybridization between these DNA probes and the large chromosomes of each isolate indicates that mini-chromosomes share common sequences with the large ones. When cloned sequences were used as probes, the majority of them gave the same results. However, two clones, a 0.16 kb *Bam*HI fragment of the 1.43 Mb mini-chromosome of strain A-17, and a 0.57 kb *Eco*RI fragment of the 1.26 Mb mini-chromosome of strain A-2, named as RMS-1 and RMS-2, respectively hybridized exclusively to mini-chromosomes of all *F. sporotrichioides* strains tested. Several closely related species were also probed with RMS-1 and RMS-2, but no hybridization signal was observed. Consequently, these probes are both mini-chromosome specific and species specific ones. Mini-chromosomes in *F. sporotrichioides* are thus mosaics of dispersed repeats and unique sequences. The finding that this mosaic structure was maintained in all genetically isolated, non-interbreeding strains strongly suggests that these structures are useful components of the genome.

É. TÁBORHEGYI and L. HORNOK

Trichothecene and zearalenone mycotoxins in *Fusarium* species

Agricultural Biotechnology Center, Gödöllő and Department of Microbiology, Agricultural University, Gödöllő, Hungary

Fusarium graminearum, *F. poae* and *F. sporotrichioides* isolates were screened for mycotoxin production. Sixteen trichothecenes and 2 zearalenones were identified and quantified by gas-liquid chromatography. *F. sporotrichioides* proved to be the most

toxic species. All strains of this fungus were able to produce T-2 toxin and all but one synthesized neosolaniol. 3-Acetyl-diacetoxyscirpenol, acetyl T-2 toxin, 4,15-diacetoxyscirpenol, HT-2 toxin, and T-2 triol were detected in most strains, while 15-monoacetoxyscirpenol and iso-T-2 toxin were identified in trace amounts and only in two isolates. There were great qualitative and quantitative differences in toxigenic capabilities among the strains. In case of the major compounds (neosolaniol and T-2 toxin) the quantitative variations predominated: neosolaniol was produced from 2.6 to 533.0 $\mu\text{g g}^{-1}$, while levels of T-2 production varied between 6.5 and 1120.0 $\mu\text{g g}^{-1}$. Differences in the trichothecene patterns were independent on the geographic or plant origins of the strains. *F. graminearum* produced high amounts of deoxynivalenol, 15-acetyldeoxynivalenol and zearalenone (79.8, 156 and 434 $\mu\text{g ml}^{-1}$, respectively), while no toxigenic isolate was found in *F. poae*, a well-documented trichothecene producing fungus.

T. EMRI, G. BARTÓK and A. SZENTIRMAI

**Effect of nitrogen source on the regulation
of the oxidative pentose phosphate shunt
in *Penicillium chrysogenum***

Department of Microbiology and Biotechnology, Kossuth Lajos University, Debrecen, Hungary

It has been known that the assimilation of ammonia and nitrate requires NADPH, and as a consequence possibly the oxidative pentose phosphate shunt is regulated by nitrogen source in several fungi. In the present study we demonstrate that the specific glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities changed when *Penicillium chrysogenum* was grown on different nitrogen sources. The specific enzyme activities in the presence of 5 mM ammonia were about 50% higher than those in the presence of 5 mM glutamate. Nitrate could also increase the specific enzyme activities if nitrate reductase had been induced by addition of small quantities of nitrate. The specific enzyme activities were not influenced by the glutamate concentration. Hence we assume that an increase in the intracellular ammonia concentration was responsible for the observed increases in the enzyme activities. This assumption was further supported by the correlation observed between the intracellular ammonia concentration and the specific enzyme activities.

E. RAHMANI and CS. DOBOLYI

**Colonization kinetics of arbuscular mycorrhiza
in the roots of different alfalfa species**

Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary

The growth of arbuscular mycorrhiza (AM) was followed in the seedlings of four alfalfa species in two soft types. The colonization rate and the number of spores in rhizosphere as quantitative parameters were investigated in pot experiment. Different AM spore types were recognized as qualitative feature in order to estimate the diversity of AM fungal populations. An intensive AM was formed in seedlings in both the Ramann's brown forest soft from Hungary and the alluvial soil from Iran at the age of eight weeks. However, the colonization rate markedly depended on the plant species and the soft type. In the Ramann's brown forest soil in the roots of *Medicago orbicularis* and *M. scutellata* coenocytic hyphae appeared at the age of four weeks and even the arbuscules and vesicles were seen at the age of six weeks. These processes were observed in *M. truncatula* and *M. sativa* only with two weeks delay. The extent of colonization was the highest in *M. scutellata* (60.6%) and the lowest in *M. sativa* (21.4%). The rate and the extent of AM colonization proved to be moderately higher in the Iranian alluvial soil than in the Ramann's brown forest soil. In the rhizosphere of all four alfalfa species a relatively high mount of AM spores (150–950 per 100 g soil) could be found in both soil types. During the eight-week experiment 2–6 spore types could be differentiated in the investigated alfalfa rhizospheres.

A. SZENDRŐI, L. G. GAZSÓ and E. J. HIDVÉGI

**Metabolization of acidophilic autotroph and heterotroph
bacteria – depyritization of the lignite from Bükkábrány**

Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary

In association with acid rain the main question is whether we can use a biodesulphurization process instead of the chemical method. Our experiments focus on getting to know the pyrite content of the lignite can be decreased by *Thiobacillus ferrooxidans* and an acidophil heterotroph bacterium *Acidophilium cryptum*. The ferro ion oxidation capacities of some *T. ferrooxidans* using mixed culture leaching the lignite from Bükkábrány have been investigated. *T. ferrooxidans* and an acidophilic heterotroph bacteria have been isolated at the area of Bükkábrány lignite mine in order to have strains with better capability for desulphurization of the Hungarian lignite.

J. HEGÓCZKI, B. JANZSÓ and Á. SUHAJDA

Production of chromium yeasts

Department of Agricultural Chemical Technology, University of Technical Sciences, Budapest, Hungary

For the normal function of living organism proper micro element supply is necessary. There is evidence that normal biological activity of insulin is dependent on a sufficient level of chromium. Since chromium is lost daily through excretion, a diet deficient in assimilable chromium will lead to glucose intolerance. It was noted that natural organic chromium complexes differed from simple inorganic chromium compounds in respect to the rate of intestinal absorption, tissue distribution, and in other effects. When chromium deficiency occurs, dietary supplementation with high levels of inorganic chromium compounds is needed to restore chromium sufficiency, as contrasted to the much lower levels of organically bound chromium that are required to counteract nutritional deficiency. Under adequate circumstances yeasts are able to take up micro elements and incorporate them into organic compounds in their cells. In the course of our investigations baker's yeast (*Saccharomyces cerevisiae*) and yeast for feed (*Candida utilis*) were grown in laboratory scale fermenter, in nutrient medium based on glucose and molasses using batch technique. A water-soluble chromium salt ($\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$) was added into the nutrient medium during the accelerating phase. The amount of addition was determined by preliminary experiments in shaking flasks cultivation by measuring the growth and micro element cultivation to avoid significant decrease in the biomass, but to attain satisfactory micro element enrichment. After cultivation the biomass was separated, washed, filtered and dried. The concentration of chromium both in the medium and the yeast were determined by Inductively Coupled Plasma Atomic Emission System (ICP-AES). This new natural micro element is more suitable for and favourable to both human and animal organism. This "biotransformed" chromium shows better absorption quotient, is less toxic, has a pleasant taste and is easier to portion in dosages and to package. Another benefit is the protein of full value and high vitamin content of the yeast.

Á. SVEICZER, B. NOVÁK and M. MITCHISON

Mechanisms of size-control in the fission yeast cell cycle

Department of Agricultural Chemical Technology, Technical University, Budapest, Hungary and Institute of Cell, Animal and Population Biology, University of Edinburgh, Scotland

The normal execution of the cell division cycle requires the coordination of cell growth and division, which is realized by size-control mechanism(s): cells are able to execute some cell cycle events only if their sizes are larger than a critical value. The mitotic control of the fission yeast *Schizosaccharomyces pombe* is a classical example of size-control, and it can be studied simply by "time-lapse microphotography". Fantes

(1977) showed that cells which were large at birth had shorter cycle times than cells which were small at birth. As a consequence larger cells grow less during their cycles than smaller ones, and the division length is independent on birth length. *Wee* mutants have no mitotic size-control and the question is how size homeostasis is maintained in these cells. We have established by analysis of numerous *wee* mutants' (*wee1.6*, *wee1.50*, Δ *wee1* and *cdc2.1w*) films that these cells have the same strict size-control as wild type cells. We suppose that this size-control operates at START event before DNA-replication. Keeping *cdc* mutants (including *cdcwee* double mutants) at restrictive temperature, we can produce cells having extremely large cell lengths. By shifting-down to permissive temperature these oversized cells quickly divide at cell lengths much larger than normal. We have established that division length is dependent on birth length, so no size control is operating in these oversized cells. However there is no sign in our data that the slope of cycle time vs. birth length graph ever reaches zero, as would be expected with a pure "timer" mechanism.

A. GELETA and A. MARÁZ

**Ethanol induced flocculation in
*Schizosaccharomyces pombe***

*Department of Microbiology and Biotechnology, University of Horticulture and Food Industry,
Budapest, Hungary*

Spontaneous aggregation of yeast cells to macroscopic clumps (flocs) is termed flocculation, which generally takes place in special growth phase of the culture. We selected a *Schizosaccharomyces pombe* strain which can ferment malic acid and shows a flocculation character from the beginning of the late exponential growth phase when the ethanol concentration is minimum 5% (v/v) in the culture medium.

The presence of Ca^{2+} ions is a prerequisite for the flocculation but Mg^{2+} Na^{+} and K^{+} ions can substitute it. Heat treatment of flocculated cells caused reversible (55 °C, 10 min) or irreversible (70 °C, 10 min) flocculation while proteases (pronase, proteinase-K, trypsin, alfa-chimotrypsin) and cell wall lytic enzymes (novozim, lyticase, hellcase) irreversibly ceased flocculation. It seems highly probable that lectin-type protein molecules are involved in flocculation, similary as in the case of some other flocculating yeast strains (e.g. *Saccharomyces cerevisiae* and *Kluveromyces bulgaricus*). Because lectins are a special class of proteins that have a high affinity for sugars, the deflocculating effect of different sugars, sugar-derivatives and polysaccharides was studied. The result shows that a galactose specific lectin is responsible for the flocculation of this *S. pombe* strain.

B. GRALLERT, B. RIBAR and M. SIPICZKI

Synthetic skip of septation in fission yeast mutants

Department of Genetics and Institute of Biology, University of Debrecen, Debrecen, Hungary

The unicellular fission yeast *Schizosaccharomyces pombe* var. *pombe* divides by fission. First a trilaminar septum is formed in the middle of the sausage-shape cells; then the middle layer of the septum is hydrolyzed by specific enzymatic reaction. The mutants *sep1-1* and *spl1-1* are defective in this hydrolysis and do not complete cytokinesis: the progeny cells remain unseparated and form, after several divisions, multiseptate mycelia (Sipiczki et al. 1993). In this report we show that the mutation *sep1-1* interacts with mutations in *wee1*. The double mutant forms mycelia in which up to 50% of the cells contain 2 to 4 nuclei. Measurements by flow cytometry and immunofluorescence staining of cytoskeleton revealed that DNA synthesis and karyokinesis are not affected but cytokinesis is frequently skipped between two cell cycles. Preliminary results suggest that this may be due to a synthetic delay of the initiation of septation.

Á. NAGY, CS. VÁGVÖLGYI, ZS. PALÁGYI and L. FERENCZY

Analysis of chromosomal DNA polymorphism of *Phaffia rhodozyma*

Chemical Works of Gedeon Richter Ltd., Budapest and Department of Microbiology, Attila József University, Szeged, Hungary

Phaffia rhodozyma synthesises astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione) as its principal carotenoid, which could be used as a colorant of feed for the aquaculture industry. Strain improvement is considerably impeded by the lack of information about the genetic organisation of this microorganism. In the present study, the pulsed-field gel electrophoresis (PFGE) technique was used to obtain data on the genome organisation of *P. rhodozyma*. A protoplast formation protocol and a method for the preparation of intact chromosome-size DNA molecules were worked out. The optimal parameters for the separation of DNA molecules were established for analyses by both OFAGE (orthogonal field alteration gel electrophoresis) and CHEF (contour clamped homogeneous electric field). The OFAGE system was able to separate appropriately either the small or the large chromosomal DNA molecules under one set of conditions. In contrast, the CHEF system effectively resolved both small and large molecules in one single run by sequential changing of the crucial running parameters. The six tested *Phaffia* strains revealed high chromosomal length polymorphisms. Seven to thirteen chromosomal bands, ranging in size from 0.83 Mb to 3.55 Mb, were resolved, giving total genome sizes of about 15.54–23.84 Mb.

Á. NAGY, CS. VÁGVÖLGYI and L. FERENCZY

**Preparation of chromosomal DNA from yeasts
of biotechnological importance**

*Chemical Works of Gedeon Richter Ltd., Budapest and Department of Microbiology, Attila József
University, Szeged, Hungary*

The use of pulsed field gel electrophoresis (PFGE) has become an efficient approach for analysing fungal genomes. Protoplast formation could be the critical step in all these procedures, as low efficiency of protoplast formation, loss and degradation of both protoplasts and DNA in the course of sample preparation, could seriously limitate their applicability. Different modifications have been worked out (e.g. liberation of chromosomal size DNA from intact cells) to overcome these drawbacks. However, in contrast to some other yeasts, we could not find the conditions for the astaxanthin-producer *Phaffia rhodozyma* to liberate chromosomal DNA from unprotoplasted cells. To avoid these problems, an efficient small-scale method was worked out for obtaining electrophoretic karyotype from this basidiomycetous yeast. This procedure involves the degradation of cell wall in the agarose matrices of the sample plugs by the combined action of two lytic enzymes, without the need for special buffer systems. Separation of DNA from these samples resulted in clearly resolved electrophoretic karyotypes. The chromosomal patterns were identical whether it was resulted in by embedment of preformed protoplasts or in situ protoplast formation. The applicability of this protocol for some other yeasts of biotechnological importance (*Hansenula*, *Kluyveromyces*, *Saccharomyces*) are also demonstrated.

J. KUCSERA, I. PFEIFFER and L. FERENCZY

Occurrence of petite mutation in *Phaffia rhodozyma*

Department of Microbiology, József Attila University, Szeged, Hungary

The red-pigmented fermentative yeast *Phaffia rhodozyma* identified by Miller currently is at the focus of interest because of its astaxanthin production. A good deal of information are available concerning the strain improvement and increasing the production. Nevertheless little is known about the genetics of this species. During our preliminary study on *Phaffia rhodozyma* strains (CBS 5905 and ATCC 24261) the occurrence of large and small colonies with different frequency was revealed on complete media. Replicating the colonies onto ethanol-containing media the small strains failed to growth. Presumably these colonies represent spontaneous petite mutants, consequently a new petite positive species was identified. Specific induction of petite mutation was carried out by ethidium bromide treatment. The efficiency of the treatment was different on the two strains. The effect of ethidium bromide can be explained by the elimination of the linear DNA plasmids from the ATCC 24261 strain

but it can not be the explanation for CBS 5905 strain where the plasmids were originally absent.

J. VARGA, CS. VÁGVÖLGYI, Á. NAGY and L. FERENCZY

Molecular characterization of *Phaffia rhodozyma* strains

Department of Microbiology, Attila József University, Szeged and Chemical Works of Gedeon Richter Ltd., Budapest, Hungary

Phaffia rhodozyma is a red-pigmented fermentative yeast, which produces the commercially important pigment astaxanthin. This pigment is also responsible for the pigmentation of salmon, crustacea and flamingos. The species is asexual, and the lack of knowledge about its genetics and variability makes strain improvement difficult. Some *P. rhodozyma* collection strains were compared as regards their isoenzyme profiles, ribosomal DNA and RAPD patterns. The isoenzyme profiles appeared to be more stable than the RAPD patterns, or the electrophoretic karyotypes determined earlier. The ribosomal DNA patterns obtained after digesting the total DNA preparations of the strains with different restriction enzymes, and hybridizing the *Aspergillus nidulans* ribosomal repeat unit to the resulting DNA smear revealed only a limited degree of variability, while RAPD analysis proved to be the most useful in differentiating these strains. The type strain of the species revealed characteristic RAPD patterns, and electrophoretic karyotypes different from those of the other strains examined. However, in spite of the high degree of variability observed, the isoenzyme data and the slightly variable ribosomal DNA hybridization profiles confirm that the strains examined represent one species.

J. SERFŐZŐ, K. HALMY, ZOLTÁN SERFŐZŐ and M. HALMY

**The tubulo-vesicular system of *Candida albicans*:
alternating ways of the cell organelle's development
and formation**

Department of Comparative Animal Physiology, L. Kossuth University, Debrecen and Mycological Laboratory, Gy. Kenézy Hospital, Debrecen, Hungary

A special built cell organelle is constructed by tubules and vesicles forming an integrated system which appeared upon the effect of imidazole and triazole drugs in the blastospores of *Candida albicans*. This organelle is hypertrophized in the presence of drugs. Formal changes made it possible to observe the main stages of development and evolution of this cell organelle. The development of tubulo-vesicular system started from a cytoplasmic vesicle containing osmiophil substances. As a result of vesicular

differentiation accompanied by with formations of membranes, the intravesicular space will be divided. The hypertrophized cell organelle blossoms like a flower bud and its typical structure constructed by tubules (or sheets) and vesicles becomes visible. Its appearance reminds us of a bunch of grapes or a cock-comb in its completely developed form. Another developmental way of the tubulo-vesicular system may start from a tubular element of blastospore's cytoplasm. In this case, the birthplace can be a cell organelle which also contains osmiophil substances.

K. HALMY, J. SERFŐZŐ, Z. SERFŐZŐ and M. HALMY

**In vitro effects of antimycotics on the phagocytosis
and killing of *Candida albicans***

*Mycological Laboratory, Gy. Kenézy Hospital, Debrecen and Department of Comparative Animal
Physiology, L. Kossuth University, Debrecen, Hungary*

The phagocyte system of the host has a decisive role in the protection of pathogenic agents. The blastospores of *Candida albicans* were phagocytized and killed by the polymorphonuclear leukocytes (PMNLs) but their effectiveness was smaller in the forms developing into filamentation. The filamental transformation of *C. albicans* is prevented by imidazole and triazole derivatives and they can promote the phagocytic and killing functions of PMNLs. In our experiment, the effect of ketokonazole (imidazole), itraconazole and fluconazole (triazoles) were studied in vitro in the same dosage (10^{-7} mol ml⁻¹) as regards the phagocytosis and killing functions of separated PMNLs arising from healthy blood donors. The cell proportion of *Candida* and leukocytes was 2:1. From the antimycotics examined, itraconazole increased the phagocytosis of PMNLs significantly in comparison with the control. The effects of ketoconazole and fluconazole were smaller. At the same time, however, the effect of ketoconazole to killing of *C. albicans* was more significant. Filamentation was inhibited by all the three drugs, therefore the number of phagosomes in PMNLs increased in contrast to the control.

G. KISS, SZ. RADVÁNYI and G. SZIGETI

Diseases caused by *Malassezia pachydermatis* in dog

Central Laboratory, University of Veterinary Science, Budapest, Hungary

The genus *Malassezia* includes two species, the human pathogen *Malassezia furfur* and the zoophil *Malassezia pachydermatis*. The latter, a commensal yeast of canine skin, is commonly isolated from the ear canal of dogs with otitis externa, from the skin of dogs with pruritic dermatitis but is commonly isolated also from the anal

sacs, rectum and vagina. *Otitis externa*. Inflammation of external ear canal is a common disease in dogs. The external auditory canal provides an ideal environment for the growing of different microorganisms by a number of predisposing factors and by the anatomy of canine ear. During a complex microbiological investigation of ear discharges *M. pachydermatis* yeast was isolated more frequently, both in pure culture and associated with bacteria. The less efficient treatments in the practice was connected with the unknown etiology of otitis externa, the lack of laboratory investigations before treatments, as well as with the lack of antimycotic effect in the available preparations. For this reason our team – based on the results of etiological investigations – has developed a new specific combination for the treatment of otitis externa. *Malassezia dermatitis*. On the basis of clinical examinations and laboratory diagnostic investigations dermatitis caused by *M. pachydermatis* was diagnosed in 25 dogs. The peanut-shaped yeast cells could easily be demonstrated by microscopic examination of scales and hair from the affected skin area. Skin lesions consist of diffuse erythema, with variable scale, alopecia and hyperpigmentation. Pruritus is a major sign and constant feature. The alterations mainly developed at the eyes and upper lips, at the nose, on the external side of ears and on the skin of abdomen. As bacteria, mainly *Staphylococcus intermedius* and ectoparasites, mainly *Demodex canis* are often associated with *M. pachydermatis*, an effective therapy should be based on complex laboratory investigation.

J. ZALA, ZS. HORVÁTH and T. NAGY

Comparative mycoserological studies

Department of Mycology, Béla Johan National Institute of Hygiene, Budapest, Hungary

Because of the bad prognosis of systemic fungal diseases and the limits of their therapy to be used, rapid and adequate methods in laboratory diagnosis are needed. Since the direct histological investigations and culture methods also are often useless there is a growing claim for serological procedures.

When choosing the suitable method and planning the tests it is very important to consider many viewpoints that may have an effect on the result. The most important of them are the predisposing factors and the state of the immune system of patients, furthermore the exogenic or endogenic origin of fungi causing disease. The secondary (opportunistic) mycosis is based on other existing illnesses which usually result in a decreased activity of the immune system. In these cases specific antibody against fungi can be found rarely. When these type of antibodies are present, they have diagnostic value only in exogenic mycosis (e.g. aspergillosis). However, in majority the opportunistic mycosis has an endogenic origin (namely candidosis). In these cases the diagnosis can be supported mainly by reactions which are more or less independent from the immune response of the patients (e.g. demonstration of fungal antigens in blood). Regarding the suitability of several serological methods available in routine mycological laboratories on patients having different illnesses, as it has been expected,

the demonstration of fungal specific antibodies showed the highest correlation in pulmonary diseases. When the patients had malignant haematological diseases (e.g. different type of leukaemia) neither test of antibodies nor those of antigens showed high positivity. The serological methods (CIE, LA) seem effective mostly in acute myeloid leukaemia.

ZS. HORVÁTH, J. ZALA, A. SZÁNTÓ, P. OSVÁTH, Á. SZÉCHI, T. NAGY,
G. MAYER, V. KARCAGI and I. FARKAS

Fungal agents seasonality and host plant specificity

Department of Mycology, B. Johan National Institute of Hygiene, Central Laboratory, Biochemical Department and Environmental Department, Ferenc Jahn Hospital, Children Pulmonological Clinic, and IX. Pulmonological Department, Szabadsághegy Children Hospital, Plant Pathology Department, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary

The main three sources of known allergenic fungi are: normal floras (*Candida albicans*, etc.) alimentary (*Saccharomyces cerevisiae*, *Candida krusei*, etc.); and environment (mainly plant pathogenic molds). The last group may occurred both indoor and outdoor. Experiments for seasonality were mainly focused on outdoor allergens. Burkard trap can partially be used for monitoring whereas the whole spectrum can be demonstrated by culture system combined with volumetric sampling from air. Seasonality of main allergens occurring mainly in the end of summer and the beginning of autumn, shows a good correlation with vegetation. *Cladosporium* occurred almost during whole year, while *Alternaria* occurred during summer and autumn in large amount. The Burkard trap is not suitable for detecting *Phoma*, for which a culture method should be developed. In asthmoid bronchitis fungal allergy is mainly diagnosed by use of skin-test and/or detection of fungal specific IgE antigen (RAST) from serum.

S. CSUKÁS, Á. LÁSZLÓ, K. OLÁH, P. SIKLIÓS, T. SZAKONYI, G. SZATHMÁRY
and I. SZILLER

Aetiologic agents of acute vulvovaginitis, with special regard to *Candida*

Institute of Microbiology, 2nd Department of Obstetrics and Gynecology, and 1st Department of Obstetrics and Gynecology, Semmelweis University Medical School, Schöpf-Mérei Ágost Hospital, St. István Hospital, Nyíró Gyula Hospital, and St. János Hospital, Budapest, Hungary

Aetiologic agents were demonstrated in 308 cases in the vaginal specimens of 400 patients with acute vulvovaginitis. *Candida* strains were isolated from 272 specimens. According to the identification of yeasts, *Candida albicans* was shown in

85% of the isolates. *Trichomonas vaginalis* was detected in 10 specimens, in 5 of them together with yeasts. There was a mixed bacterial flora in 24, and *Neisseria* strains were present in 2 specimens. After 6 days local clotrimazol treatment of 137 patients, control was made on the 14th day. Yeasts were isolated in 21 specimens. Another control of the negative patients was performed on the 40th day and *Candida* was isolated in 6 specimens. A 6 days local miconazol treatment was performed with 135 patients. On the 14th day the control analysis showed 16 yeast positive cases, and on the 40th day carrier states were demonstrated in 15 patients. In 50 cases the first isolated and reisolated yeast strains proved to be identical. The in vitro demonstration of antimycotic effect is a difficult problem. Imidazole derivates significantly differed in mycostatic action for strains of the patients unsuccessfully treated. Pimaricin proved to have a strong mycocidal effect to all *Candida* strains.

T. NAGY, E. K. NOVÁK, J. ZALA and ZS. HORVÁTH

Computer aided Expert Systems usage in mycology

Mycological Department, B. Johan National Institute of Hygiene, Budapest, Hungary

Development of Expert Systems (ES) and analyzing of them in medical research requires a similar development in the connected interdisciplinal fields (e.g. mycology), too. A reliable ES can only be realized both on theoretical (mathematical model-reality, validity, etc.) and practical (necessity-possibility) point of view. Both quantitative and qualitative considerations should be taken for a suitable data structure. From the data base (cf. literature) into a new data base (ES) can be made by programme oriented (syntactical analyses-machine translation) or by experts knowledge adapted to ES. The selected mathematical model (deterministical, stochastic-e.g. fuzzy theory) requires thorough preparations in the complex mycological field. EXPERT SYSTEM programme for the identification of pathogenic fungi has been developed for the mycological routine work. EXPERT(s) can define a special graph with SELECTION LISTS, TEXTs, PICTUREs for the USERS.

ERRATUM

EXAMINATION OF VEROCYTOTOXIN PRODUCING CAPACITY AND DETERMINATION OF THE PRESENCE OF SHIGA-LIKE TOXIN GENES IN HUMAN *ESCHERICHIA COLI* ISOLATES

I. TÓTH, VERONIKA KARCAI, B. NAGY, I. GADÓ and HEDDA MILCH

*B. Johan National Institute of Hygiene, Budapest, and Veterinary Medical Research Institute
of the Hungarian Academy of Sciences, Budapest, Hungary*

Vol. 41, no. 3, p. 259, paragraph 1, line 12: "nonconvertible" should read
"nonconverting."

Page 260, paragraph 2, line 4: "shigella-like" should read "Shiga-like."

Page 261, paragraph 4, line 1: "~60 MDa" should be deleted

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Acta Microbiologica et Immunologica Hungarica

VOLUME 42, NUMBER 2, 1995

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ACTA MICROBIOL. ET IMMUNOL. HUNG. 42 (2) 141-236 (1995) HU ISSN 1217-8950

ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN
ACADEMY OF SCIENCES

Acta Microbiologica et Immunologica Hungarica publishes reviews and original papers on microbiology and immunology in English

Acta Microbiologica et Immunologica Hungarica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ
Publishing House of the Hungarian Academy of Sciences
H-1117 Budapest, Prielle K. u. 19-35

Manuscripts and editorial correspondence should be addressed to

Acta Microbiologica et Immunologica Hungarica
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Subscription price for Volume 42 (1995) in 4 issues US\$ 98, including normal postage, airmail delivery US\$ 20.00.

Acta Microbiologica et Immunologica Hungarica is abstracted/indexed in Abstracts of World Medicine, Biological Abstracts, Chemical Abstracts, Chemie-Information, Current Contents-Life Sciences, Excerpta Medica database (EMBASE), Index Medicus

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CONTENTS

Evolutionary Aspects, and Taxonomic Definition of Viruses together with Mobile Extrachromosomal Elements of Relative Autonomy into a Special Highest Rank Taxon (A Review)	
<i>Berencsi, Gy., Bánrévi, A., Takács, M., Lengyel, A., Nász, I.</i>	141
Nutrient Optimization for Production of Broad Spectrum Antibiotic by <i>Streptomyces antibioticus</i> Sr _{15.4}	
<i>Haque, S. F., Sen, S. K., Pal, S. C.</i>	155
In Vitro Suppression of Anti-TSH Receptor Antibody by Autologous Anti-Idiotypic Antibody in Patients with Graves' Disease	
<i>Balázs, Cs., Molnár, I.</i>	163
Effects of Some Heavy Metals on Growth, Pigment and Antibiotic Production by <i>Streptomyces galbus</i>	
<i>Raytapadar, S., Datta, R., Paul, A. K.</i>	171
Altered Humoral Immunity against Cytomegalovirus and Epstein-Barr Virus without Detectable Virus Antigens and Virus-DNA in the Glomeruli of Patients with IgA Nephropathy in Remission Phase	
<i>Nagy, J., Haikin, H., Sarov, B., Háber, Á., Kun, L., Sarov, I.</i>	179
Longitudinal Immunological Follow-up of HIV Infected Haemophiliacs in Hungary	
<i>Ujhelyi, E., Králl, G., Zimonyi, I., Bánhegyi, D., Hollán, S. R., Horváth, A., Fuchs, D., Wachter, H., Berkessy, S., Füst, G.</i>	189
Remarks on the Appearance of "Violette" on Cantal Cheese (A Note)	
<i>Chaballier, C., Ratomahenina, R., Galzy, P., Dieu, B.</i>	199
Immunomodulating Effect of Acridine Tautomers on Eukaryotic Cells	
<i>Petri, I. B., Berek, I., Galy, A.-M., Barbe, J., Berek, L., Molnár, J.</i>	203
The Effect of Vasopressin on the Phosphoinositides of <i>Tetrahymena pyriformis</i> . Relationship with Glycogen Content	
<i>László, V., Csaba, G.</i>	209
A Model for Testing Drug Susceptibility of <i>Pseudomonas aeruginosa</i> and <i>Staphylococcus aureus</i> Grown in Biofilms on Medical Devices	
<i>Kétyi, I.</i>	215
Biofilms Produced by <i>Pseudomonas aeruginosa</i> and by <i>Staphylococcus aureus</i> on Model Medical Devices	
<i>Kétyi, I.</i>	221
Dehydroepiandrosterone Modulates the Spontaneous and IL-6 Stimulated Fibrinogen Production of Human Hepatoma Cells	
<i>Fehér, K. G., Rákász, É., Bíró, J., Falus, A.</i>	229
Detection of Hepatitis C Virus (HCV) by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (A Note)	
<i>Falus, A., Hajnal, A.</i>	235

PRINTED IN HUNGARY

Akadémiai Kiadó és Nyomda, Budapest

**EVOLUTIONARY ASPECTS, AND TAXONOMIC
DEFINITION OF VIRUSES TOGETHER WITH MOBILE
EXTRACHROMOSOMAL ELEMENTS OF RELATIVE
AUTONOMY INTO A SPECIAL HIGHEST RANK
TAXON
(A REVIEW)**

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(Received October 7, 1994)

(Accepted October 19, 1994)

Introduction	141
The problem of intracellular parasites, and symbionts	142
Taxonomic importance of cell organelles and bacterial sporulation	143
Taxonomic importance of the eclips	143
Infectious gene products, or mobile genetic elements, which cannot be classified using the accepted taxonomic criteria.....	144
Evolutionary predecessors and/or ancient companions of the known living world in the light of taxonomic definition of viruses, and mobile genetic elements.....	145
Why DNA viruses are not listed among the elements of perished ancient hosts?.....	146
Modular evolution of bacteriophages, viruses, and other extrachromosomal elements	147
The problem of viral latency as a genetically regulated prolonged eclips phase of viruses of vertebrates	148
Conclusions	148
Acknowledgements.....	149
References.....	149

Introduction

It has been proposed about 18 years ago, and published several years later [1], that in addition to the structural properties of viruses characteristics of eclipsed and integrated states of the viral genomes have to be included into the taxonomic definition

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of viruses. The only description of the extracellular virus may never fulfil the requirements of the taxonomic definition [2].

Even in the most recent publication on classification and nomenclature of viruses [3/a] 72 keys have been listed which can be used for the identification of a virus, nevertheless, only the host, and the way of transmission provide information about the natural cycles of them.

The number of conflicting evidences are growing continuously because of the application of general taxonomical entities (class, order, genus, species) for the classification of viruses. The authors suggest, that controversies might be resolved by the united taxonomical definition of mobile extrachromosomal elements of relative autonomy together with viruses, and other mobile and/or infectious gene products.

The problem of intracellular parasites, and symbionts

Several species of *Chlamydozoaceae* [4, 5], and *Rickettsiaceae* are obligatory parasites of mammals, and arthropodes with the exception of *Rochalimeae*. Protozoa such as *Trypanosoma cruzi* and *Plasmodium* are also obligate parasites of the human species, mammals, and arthropodes. *Legionella* species are able to grow as intracellular parasites of both protozoa, and macrophages of mammals. Tissue phases of leishmania infections include intracellular replication of the flagellata [6]. In addition to these medical precedents innumerable exemplars of endosymbionts can be identified in nature [7, 8]. Protozoa are regularly consuming bacteria [9, 10] in their natural environment, which are carriers of viruses and mobile genetic elements. Highly developed regulatory mechanisms lead to the formation of *Rhizobium meliloti*'s nitrogen-fixing nodules within the root tissues of *Medicago*, *Melilotus* and *Trigonella* host plants [11]. *Agrobacterium tumefaciens* and host plant systems have been characterized, and utilized for genetic modification of plants during the last decade [12], indicating that bacteria may transfer genes even into plant cells. In other systems viruses can be transmitted passively, without replication in the fungal vector [13, 14].

No comprehensive list of the possible symbiotic and parasitic systems in nature can be given in a review article. The above exemplars are supposed to illustrate that no taxonomic problems are raised at the level of symbiosis and intracellular parasitism, *since well defined host species (or their differentiated cells) are invaded and utilized by a different, well defined taxonomic entity.*

A taxonomic problem is created, when the intracellular alga living in symbiosis with a host cell (*Chlorella* from *Hydra viridis* [15]) becomes infected with a virus. The taxonomic definition of other extrachromosomal elements, which can be present within the intracellular parasites or symbionts may cause similar problems, since kinetoplasts are present in *Flagellata* [16], linear episomes [17] may appear in them. Megaplasmids or plasmids [11, 18], and temperate bacteriophages [19] can be carried by symbiotic bacteria, and the number of transposable and insertion elements in different participants of heterogeneous natural populations also have to be taken into account [20]. Even mitochondria may carry integrated or autonomous plasmids in certain species [21, 22].

It is difficult to understand, that integrated temperate bacteriophages, and endogenous retroviruses are classified as families, genera and species of viruses in contrast to all other mobile genetic elements (summarized first by Mims, 1981 [23]) which can be transmitted vertically even via the germ-line.

Taxonomic importance of cell organelles and bacterial sporulation

Experimental evidences have been collected during several decades, indicating, that mitochondria, and different populations of chloroplasts possess prokaryotic ancestors [24–31]. Although the possible taxonomic positions of the ancient precursors of chloroplasts and mitochondria had been identified, nobody intended to create separate taxonomic entities for these organelles, nevertheless, they can be readily identified as cytoplasmic extrachromosomal elements of relative autonomy [32–34].

If the extracellular, condensed forms of viruses are considered to be definitive for classification, endospores of bacteria should be classified as a separate taxonomic entity. Formation of bacterial spores are the results of special regulatory processes, which result in the production of an asymmetrically condensed, dehydrated latent cell, which possesses a specific trigger system for recovery. Recently the episomal nature of a germination gene has been also suggested [35]. Temperate bacteriophages, transposons may be present in the chromosomal material of endospores, too. Nobody had up to now the idea to classify spores into separate taxa from those of the vegetative bacteria.

In contrast to cell organelles, and endospores of Gram-positive bacteria, poxviruses and baculoviruses are classified into separate families, subfamilies, genera and species [3/b/d] of viruses, in spite of the fact, that all of them are obligate cytoplasmic or nuclear parasites. They can only produce immediate-early (m)RNA molecules in the particle form, several members (*molluscum contagiosum*) do not kill the host cells, but stimulate their multiplication by the production of an epidermal growth factor-like substance [36, 37], and similar to the DNAs of cell organelles they are possessing a series of genes (*vaccine virus*) homologous to those of both arthropodes, and different mammals [38], and they are at least as dependent on the host cell metabolism as cell organelles. The infectious nature and extracellular survival of poxviruses, are not sufficient for a different concept of classification. These problems have been raised in several publications during the last 15 years [1, 21, 39–44].

Taxonomic importance of the eclips

There is an essential difference between intracellular parasites, and cell organelles in comparison to viruses: *the eclips phase or the nuclear or cytoplasmic phase of their replication*. No membranous or cell-wall barriere is separating viruses for shorter or longer periods of their biological cycles from the molecular machinery of the hosts. During these intracellular phases of replication mobile genetic elements and other infectious gene products are closer related to viruses than any other forms of

intracellular passengers of nature, therefore, the authors believe that *this phase has to be taken into account, when taxonomic definition, and classification of movable elements and viruses is correctly accomplished.*

It has to be emphasized that this intimate molecular phase of viral replication can occur within several hundreds of different host species [41–47] while other viruses can be transmitted passively or productively by taxonomically very different vectors indicating the necessity of additional criteria for classification than components of the extracellular virus particles [48].

Infectious gene products, or mobile genetic elements, which cannot be classified using the accepted taxonomic criteria

Infectious proteins are the products of genetically altered mammalian or probably eukaryotic genes (scrapie-type *prion proteins* [49]). Protein products of normal prion genes of mammals are non-infectious. In familiar genetic diseases (Creutzfeldt-Jacob and Gerstmann-Straussler-Scheinker syndromes) with or without the participation of small RNA molecules the protein becomes infectious. Recently a possible molecular basis has been suggested for the explanation of this controversial property [50–53].

Infectious circular RNA molecules, without protein-coding capacity – the viroids [54] – are also missing from the actual classification, in spite of the fact, that these are true extrachromosomal elements [55], transcribed by the hosts' RNA polymerase II [56] resembling to small nuclear RNAs of eukaryotic cells [57] in many respects [58], and to virus-coded small RNA populations of known [59] or unknown regulatory functions [60, 61].

Infectious circular RNA with coding capacity (defined by several authors as *virinos*) are of essential importance from the viewpoint of taxonomic definition. *Hepatitis D virus* of humans and different animals (HDV) seems to be a true virus, although of defective nature, but possessing the rolling-circle RNA replication mechanism resembling to that of viroids [62]. Hepatitis D virus could not be classified by ICTV, in spite of the fact, that its morphological properties are corresponding to a true cubical virus. The genome has, however, characteristic properties of viroids, including "self-splicing" ability. The taxonomic problem is further complicated by the fact, that HDV is replicating equally well in human, woodchuck, duck and ground squirrel hosts, depending on the persistent infection with species-specific hepatitis B virus, which provide the envelope for it. It has to be mentioned that one of the helper hepadna viruses (duck hepatitis B virus) has been classified into a separate "genus of the family" [3/c].

Several other intermediate structures may support the specific idea of classification of infectious extracellular elements, together with viruses. Members of the yeast *Ty-1 transposons* code for conserved, capsomere-like proteins. The transposition process includes the formation of virus-like particles carrying reverse transcriptase, and even packaging signals can be identified on the transcripts [63, 64]. Nevertheless, these particles can never exit from the cells. The role of the intracellular capsids may have the function to prevent diffusion of the substances necessary for Ty-1

synthesis, thus providing a transient compartment for the transposons within the cells [65], and a recent observation of Oroszlán, S. (1993, personal communication) that retroviral proteases are activated following the penetration into the new target cells, while their internal capsid remains intact during reverse transcription in order to transport the provirus into the nuclei, emphasizes the importance of capsid compartments even in the case of recently evolved retroviruses.

The *satellite genes of fungal killer systems* [21, 66, 67/a, 67/b] belong to this group, too. Their taxonomic classification as accidental passengers of *Totiviridae* [3/e] seems to confuse the natural history of these elements. It has to be declared, that no strict boundary can be drawn between transposons, copia-like elements and retroviruses [20], and the recent discovery of msDNA elements in prokaryotes emphasize the difficulties [68]. The chromatin structure of the mammalian chromosomes provides a series of arguments, that viral and non-viral retroposons have essential role in the evolution of the genomes [69–71]. The evolutionary importance of copia, delta, gipsy and Ty elements had been emphasized, too [21, 72, 73]. Movable genetic elements have been suggested to be evolutionary ancestors of retroviruses [74, 75].

Recently groups of linear dsDNA elements (integrons) and a different group including, the linear DNA genomes of DNA genomes and adenoviruses (invertrons) have been separated, which indicate the evolutionary relationships emphasized by this work [22, 76–80].

Evolutionary predecessors and/or ancient companions of the known living world in the light of taxonomic definition of viruses, and mobile genetic elements

The discovery of RNA splicing offered a new, and definitive argument for those who advocated [81] the existence of living systems, possessing RNA as the only genetic material (reevaluated first by Reanne [82]). The nucleic acids of many viruses are absent from uninfected host cells, and if present, only in the form of extrachromosomal or mobile genetic elements.

The most important relicts seem to be the *dsRNA genomes, of different segmentation*. Prokaryotes, fungi, plants, invertebrates, fishes, and mammals possess dsRNA genomes [83–86]. Yeasts possess even extrachromosomal elements coding for killer proteins, which can be transmitted by dsRNA viruses in the form of satellite RNA molecules [66, 67/a, 67/b]. The fact, that bacteriophages called *Cystoviruses* possess envelope cannot exclude the evolutionary relationship to eukaryotic dsRNA viruses, since recently the formation of envelope has been discovered in connection with budding into endoplasmic reticulum as a transient stage of morphogenesis [87].

Viruses of negative sense RNA genomes which possess unique replication and transcription mechanisms [88–93], belong to the second group including borna-, filo-, orthomyxo-, paramyxo-, and rhabdoviruses of mammals, invertebrates, and plants [93–98].

Viruses of negative, ambisense and/or segmented genomes are probably independent of the previous group (bunyaviruses, tenuiviruses) since the replication and transcription strategies seem to be different [3, 47, 99, 100]. The group of arenaviruses might be closely related to this highest taxon [99, 101, 102].

Viruses of positive stranded RNA genomes, which are transcribed into several subgenomic mRNA molecules expressing individual proteins (coronaviruses, lactate dehydrogenase virus, and equine arteritis virus) with or without leader-primed synthesis of subgenomic RNAs [99, 103–105], can be considered as unusual replication strategy as far as the uni- or multicellular world is concerned.

The fundamental taxonomic property of these groups of viruses is the genome, structure and replication strategy. This kind of classification has been suggested many years ago by Thomas, Jr. and MacHattie [106] for DNA and by Baltimore [75] for RNA viruses.

Why DNA viruses are not listed among the elements of perished ancient hosts?

Recently *ssDNA plasmids* have been discovered [107], which may or may not be related to *ssDNA bacteriophages* or *parvoviruses*. The lack of information does not allow drawing conclusions.

Hepadna viruses belong to an unusual group of viruses carrying similarities to the group of *cauliflower mosaic virus*. Members of both virus groups possess open circular dsDNA genomes with single-strand discontinuities [108, 109]. It has been shown for the human hepatitis B virus, that the encapsidation signal is located on the overlapping large RNA transcript, and the DNA of both virus groups is synthesized by reverse transcription preceding the burst and/or budding from the cells [110, 111]. The DNA polymerases of both viruses are single-subunit enzymes, which seem to be related to both RNA-dependent RNA-polymerases, and reverse transcriptases [112]. Sequence data suggest, that both polymerases are carrying oligopeptide motives resembling to those of different reverse transcriptases [113].

It is concluded by us that these two groups of viruses are descendants of retroviruses, which are able to replicate without covalent integration into the chromosomal DNA of the hosts, thus escaping *cis* regulatory influences of the host cells.

The DNA of adenoviruses, related bacteriophages and invertrons seem to be also unique in the present world of life [77, 78, 114–116]. The main characteristics of the replication strategy is the initiation of DNA replication by a terminal protein at the inverted terminal repeats followed by asymmetrical elongation [117]. The principal feature of asymmetrical elongation eliminates the production of Okazaki fragments in the case of both strands of the linear dsDNA genomes. At this point the world of prokaryotes offers an analogy for the DNA-replication mechanism of invertron-like elements. Protein primed initiation, and asymmetrical replication occurs during bacterial conjugation [118, 119] and probably in the case of bacterial transformation, too [120, 121]. The fact, that PRD1-related bacteriophages can infect many hosts by a plasmid-dependent manner [122] seems to be an important argument for the evolution of these viruses from a plasmid – (i.e. transfer factor) – dependent process, bacterial conjugation. Although, the predecessors of these DNA virus groups, and the linear mitochondrial plasmids [116] have to be analysed further, it can be suggested, that these groups (hepadna and invertrons) have to be classified into two separate highest

rank taxa in spite of the different particle morphologies of the different viral members within the groups [3].

Modular evolution of bacteriophages, viruses, and other extrachromosomal elements

The theory of modular evolution has been put forward as early as 1980 [123]. An infinite number of publications suggested, and proved that the basic element of evolution is a domain and/or a gene of the different organisms [124]. RNA polymerases of viruses possess completely different trees of sequence relationship than the accepted system of classification based on the properties of extracellular virus particles [112, 125, 126]. The rubella virus and human hepatitis E virus seem to be closely related on the basis of sequence data of RNA polymerases [127], nevertheless, genome structures, virus particles etc. belong to unrelated taxonomical entities [128]. Viruses of ssRNA and dsRNA genomes are classified into the same medium rank taxa (i.e. potyviruses and hypoviruses or sobemoviruses and spheridiplomnaviruses) on the basis of RNA polymerases [43]. Methyltransferase domains are similar in flaviviruses and reoviruses [38]. Different domains of genes have been found in prokaryotic, eukaryotic chromosomal, mitochondrial, and extrachromosomal elements [79, 129, 130].

The modular evolution of genetic elements also suggest that the virus particle cannot be the basis for virus classification, since different genomes can be encapsidated or assembled into different capsids, or envelopes. The best examples for these are various satellite viruses, associated viruses, artificial and natural pseudotypes, and defective interfering particles [41, 45, 62, 83, 131]. An outstanding example for the modular evolution is provided by tectiviruses and adenoviruses. PRD1-related bacteriophages [3, 122] possess apical fibres and belong to the promiscuous phages, since the bacterial sensitivity is only coded for by plasmids. Their genomes have terminal proteins and the genome structure seem to be similar to those called invertrons including adenoviruses [46, 77]. Nevertheless, bacteriophages Fi15, Fi29, and fungal viruses (rhizidoviruses) possess icosahedral, lipid-free capsids with and without tails [46, 132, 133]. Adenoviruses with the complicated assembly processes represent a different structure with fibers, and histon-like core-proteins [134].

Similar to the complicated evolution of retroposons, msDNA and retroviruses discussed earlier, the evolution of bacteriophages and plasmids coding for bacteriocins possess common features. Several genes coding for bacteriocins are believed to be descendants or ancestors of bacteriophages [135, 136].

An interesting observation has been reported recently in connection with the 5' untranslated region of human hepatitis C virus RNA. Five short open reading frames (ORFs potentially coding for 10–20 amino acids) are present within the 341 nucleotide-long region [137], which seem to be related to the 5'-end ORFs in *Escherichia coli* and yeast mRNAs responsible for attenuation [138]. If a similar function can be verified in pestiviruses, this will be the best verification of the modular evolution of mobile extrachromosomal elements including viruses.

The problem of viral latency as a genetically regulated prolonged eclipse phase of viruses of vertebrates

A series of viruses (iridoviruses, retroviruses, adenoviruses and herpesviruses etc.) are able to remain lifelong present latently or persistently in certain cells of the host organism. Latent adenoviruses possess specific genes to prevent apoptosis of the host cells, and then reduce the expression of MHC class I mRNA and glycosylation of MHC class I heavy chains by both E1 and E3 gene products, and eliminate receptors for epidermal growth factor in order to prevent both DNA replication of host cells and adenovirus genomes [139]. This strategy prevents the elimination of latently adenovirus-infected cells by cytotoxic immunity. It has to be emphasized that the macroorganism is persistently infected, since time-to-time a certain number of virus-infected cells escape immunomodulation, and release virions. Nevertheless, the majority of adenovirus carrier cells remain lifelong latently infected.

Herpesviruses are able to anchor their DNA to the cellular matrix [140] similar to nuclear episomes (for example rDNA) in order to become distributed equally in the sister cells following cell division [141]. The latency is possible in different host cells neurones, B-, and T-lymphocytes. The site of latency of several herpesviruses are not yet known.

In the case of Epstein-Barr virus completely different mechanisms of DNA replication evolved in latently infected cells using *oriP* as origin and termination of the replication of circular molecules [142–145]. In contrast to this the *oriLyt(s)* are used in productively infected cells initiating DNA replication by the rolling circle mechanism [143].

Different levels of latency have been registered [146]. The immune defense mechanisms were shown to be down regulated by different mechanisms. EBNA-1 was found not to be immunogenic, apoptosis is inhibited by LMP-1 specific induction of *bcl-2*. EBNA-2 blocks interferon induction. The HCL class I and II expression, and that of other host cell membrane proteins (LFA-3 and ICAM-1) is indirectly down regulated in Burkitt lymphoma cells. Expression of host cell genes IL2 and interferon gamma are inhibited by viral gene products (IL10-related BCRF1), and the small nuclear RNA related RNAs in both EBV and *H. saimiri* possess additional inhibitory function(s) [61, 146–148]. The regulatory mechanisms are complicated, and involve mutual up- or down regulations with different virus-host systems, or doubly infected cells i.e. HIV and herpes or adenoviruses [149, 150].

It may be suggested that viral genomes in latently infected cells and persistently infected macroorganisms are much more related to plasmids, episomes or other mobile extrachromosomal elements, than to mitochondria and/or extracellular virions.

Conclusions

Recent experiments, and results of gene technology revealed that the exchange of genetic material is continuously proceeding under both natural and laboratory conditions [151–153]. In connection with the exchange of genetic information, viruses,

and extrachromosomal elements modify the genetic material of their hosts, and can be considered as driving force of the evolution [14, 69, 71].

In contrast to the accepted classification of viruses [3, 154] it would be more advantageous to define the viruses as mobile genetic elements of relative autonomy, which possess also extracellular forms [1].

The modular evolution of viruses may affect in addition to the viral enzymes [43, 127], structural proteins of the capsids, and envelopes, too. Characteristics of viruses has to be used as basis of the classification, which are dominant in both extracellular, and eclipsed viruses.

When the properties of intracellular replicating, or latent viruses are also taken into account as keys of identification, two conclusions may be drawn:

(1) Viruses cannot be separated from the taxonomic entities of infectious genetic and extrachromosomal elements [20, 76, 77, 116].

(2) Taxa applied for the host organisms cannot be applied for the intracellular forms of viruses, since endogeneous retroviruses or latent herpesviruses etc. exist as integrons or minichromosomes of the host cells.

New highest, medium, and lower rank taxa have to be suggested for the classification of mobile extrachromosomal elements including viruses, based on properties discussed in the previous sections. The mechanisms for the conservation of the termini of the genomes is an additional key, which can be used for the development of different entities.

The nature is so rich in mobile extrachromosomal elements and viruses, that a specific suggestion for taxonomical entities can be made only by a competent expert group of the International Committee on Taxonomy of Viruses.

Acknowledgements. The authors thank for helpful discussions and for complementary references to Susanne Berek, Claire Berencsi, Eva Gönczöl, George Fejér, Susanne Horváth, Alexi Kiss, Anna Maráz, János Minárovits, Zsolt Ruzsics. The authors have been the recipients of the grants 1-3/1991 from the Scientific Research Council, Hungarian Ministry of Social Welfare, and the grant No. 785/1991 from the Hungarian Research Fund (OTKA).

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NUTRIENT OPTIMIZATION FOR PRODUCTION OF BROAD SPECTRUM ANTIBIOTIC BY *STREPTOMYCES ANTIBIOTICUS* Sr_{15.4}

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(Received July 29, 1993)

(Accepted March 18, 1994)

Antibiotic production by *Streptomyces antibioticus* Sr_{15.4} was studied under various cultural conditions. During nutrient optimization it was found that the strain utilized glycerol as the best source of carbon at 1.044 molar level, and 0.020 molar arginine as the best source of nitrogen. The strain exhibited significant enhancement in antibiotic production when grown at pH 6.8.

Ever since Waksman discovered actinomycin in 1940 and streptomycin in 1944, antibiotic production has emerged as a rapidly expanding branch of industrial microbiology. *Streptomyces* has received attention as the tool for this purpose. Innumerable isolates of *Streptomyces* from soil samples, taken from all parts of the world, have been systematically screened in the last three decades and have yielded more than 55% of the therapeutically useful antibiotics. Newly screened species and their products are daily added to the list. During a survey of new potent producers, *Streptomyces antibioticus* Sr_{15.4} [1] was isolated. This paper reports some aspects of cultural conditions for the production of antibiotic by the isolate.

Materials and methods

Microorganism used. The microorganism used in this investigation is a soil isolate and has been identified as *S. antibioticus* Sr_{15.4} [1]. The stock culture is maintained in agar slants at 4 °C in a refrigerator.

Cultivation procedure. The selected strain was tested in thirteen different media. For further use, the Arginine Glycerol Salt (AGS) medium was chosen: (g/l) arginine monohydrochloride, 1.0; glycerol, 12.5; K₂HPO₄, 1.0; NaCl, 1.0; MgSO₄·7H₂O, Fe₂(SO₄)₃·6H₂O, 0.010; CuSO₄·5H₂O, 0.001; ZnSO₄·7H₂O, 0.001; MnSO₄·H₂O, 0.001, pH 6.9–7.1.

In the supplementation experiment the stock solution of Casamino acid and Yeast extract were separately sterilized and added to the medium aseptically as required.

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The carbon sources were also sterilized separately. For the regulation of pH, phosphate buffer was used. Submerged culturing lasted for 5 days at 30 ± 1 °C. Growth of the organism was measured by dry weight.

Assay method. To assay the antibiotic activity, the filtered culture broth was tested by agar-cup plate method [2] using *Escherichia coli* and *Bacillus subtilis* as test organisms.

Table I

Growth and antibiotic yield of Sr_{15.4} on different culture media

Sl. No.	Culture media (broth)	Growth (mg dry wt/20 ml)	Antibiotic yield (dia. of inhibition zone, mm)	
			A	B
1.	Pridham & Gottlieb's medium [3]	38	—	—
2.	Lindenbein's synthetic medium [4]	45	12	11
3.	Glycerol-asparagine [5]	48	17	14
4.	Starch medium [5]	22	—	—
5.	Gause mineral medium [6]	31	20	10
6.	Nutrient medium [7]	23	10	8
7.	YK medium [8]	42	9	9
8.	Arginine Glycerol Salt medium [9]	39	26	20
9.	Corn-meal medium [10]	54	18	9
10.	CM medium [11]	23	19	15
11.	Casein hydrolysate peptone medium	127	14	13
12.	Soya bean meal medium	167	27	21
13.	Straw infusion medium	68	15	13

Incubation: 5 days at 30 ± 1 °C; assay: 0.12 ml of culture filtrate/cup (7 mm dia.), each value represents arithmetic mean of triplicate flasks; A = *E. coli*, B = *B. subtilis*

Results

It has been found (Table I) that among the thirteen different media tested, the synthetic media of Arginine Glycerol Salt (AGS) of El-Nakeeb and Lechevalier [9] and soya bean meal among the organic media are the most suitable for antibiotic yield of *S. antibioticus* Sr_{15.4}. However vigorous growth was observed in organic media, but the production did not exceed that of synthetic media.

To find out whether supplementation could effect the antibiotic yield, the selected AGS medium was further supplemented with casein hydrolysate and yeast extract in varying concentration and combination. Experimental results in Table II interestingly showed that when casein hydrolysate or yeast extract were added separately the growth increased over the control, but the production remained unchanged. But when casein hydrolysate and yeast extract were added simultaneously

at a proportion of 0.05% and 0.1% respectively, then both production and growth increased.

Table II

Effect of supplementation on growth and antibiotic yield

Sl. No	Supplementation	Growth (mg dry wt/20 ml)	Antibiotic yield (dia. of inhibition zone, mm)	
			A	B
1	Casein hydrolysate 0.1%	42	20	17
2	0.2%	53	18	16
3	0.3%	61	17	16
4	0.4%	77	16	15
5	0.5%	78	12	11
6	0.6%	79	11	10
7	0.7%	93	11	10
8	0.8%	100	11	10
9	0.9%	107	10	9
10	1.0%	111	9	9
11	Yeast extract 0.1%	52	16	13
12	0.2%	54	17	13
13	0.3%	56	18	14
14	0.4%	58	13	11
15	0.5%	60	13	11
16	0.6%	61	13	10
17	0.7%	63	12	10
18	0.8%	67	12	10
19	0.9%	71	11	9
20	1.0%	77	10	9
21	Casein hydrolysate : Yeast extract (0.1% : 0.05%)	50	17	13
22	Casein hydrolysate : Yeast extract (0.05% : 0.1%)	55	27	24
23	Control (without supplementation)	39	24	22

Incubation: 5 days at 30 ± 1 °C; assay: 0.12 ml of culture filtrate/cup (7 mm dia.), each value represents arithmetic mean of triplicate flasks; A = *E. coli*; B = *B. subtilis*

Nine carbon sources were tested to find out the most suitable carbon source by changing the normal carbon source of the selected medium. In this experiment moderate growth and antibiotic yield were found in glycerol, glucose, fructose, mannitol and sucrose. Growth as well as yield were insignificant with other carbon

sources. However, glycerol emerged as the best source of stimulator of growth and production for the isolate (Table III). In the per cent variation experiment (Fig. 1) it was found that 1.044 M glycerol was the most suitable for growth and antibiotic yield.

Table III

Effect of different carbon sources on growth and antibiotic yield

Carbon sources (0.626% carbon)	Growth (mg dry wt/20 ml)	Antibiotic yield (dia. of inhibition zone, mm)	
		A	B
1. Glycerol	39	24	22
2. D-Glucose	31	21	18
3. D-Xylose	12	8	8
4. L-Arabinose	16	8	8
5. L-Rhamnose	12	8	8
6. D-Fructose	25	8	8
7. D-Galactose	12	8	8
8. D-Mannitol	30	10	8
9. Sucrose	32	10	9
10. Control (Without C source)	—	—	—

Incubation: 5 days at 30 ± 1 °C; assay: 0.12 ml of culture filtrate/cup (7 mm dia.), each value represents arithmetic mean of triplicate flasks; A = *E. coli*; B = *B. subtilis*

Table IV

Effect of different nitrogen sources on growth and antibiotic yield

Nitrogen sources (0.032% N ₂)	Growth (mg dry wt/20 ml)	Antibiotic yield (dia. of inhibition zone, mm)	
		A	B
1. Arginine	38	24	22
2. Urea	29	22	19
3. Ammonium sulfate	21	8	8
4. Ammonium chloride	25	8	8
5. Ammonium nitrate	28	8	8
6. Sodium nitrate	22	12	10
7. Potassium nitrate	29	8	8
10. Control (Without N source)	20	—	—

Incubation: 5 days at 30 ± 1 °C; assay: 0.12 ml of culture filtrate/cup (7 mm dia.), each value represents arithmetic mean of triplicate flasks; A = *E. coli*; B = *B. subtilis*

To check whether the alternative nitrogen source can impose any effect, six different kinds of nitrogen compound were tested (Table IV) replacing the original nitrogen source of the basal medium with 0.174 M glycerol as carbon source. Each nitrogen compound was added, to a nitrogen content equivalent to that of arginine present in the basal medium. Arginine was the best for *S. antibioticus* Sr_{15.4}.

However, it was observed (Fig. 2) that arginine was the best yield promoter at 0.020 M. Though the antibiotic production in the presence of urea was not too less than that of arginine, but if growth and yield parameters were concerned, arginine proved its superiority.

To find out the optimum pH for antibiotic yield, the isolate Sr_{15.4} was grown at different pH using potassium phosphate buffer at a pH range between 6.0–8.0. The result (Fig. 3) showed that at pH 6.8, *S. antibioticus* Sr_{15.4} showed its maximum antibiotic yield. The antibiotic production decreased with the increase in pH value.

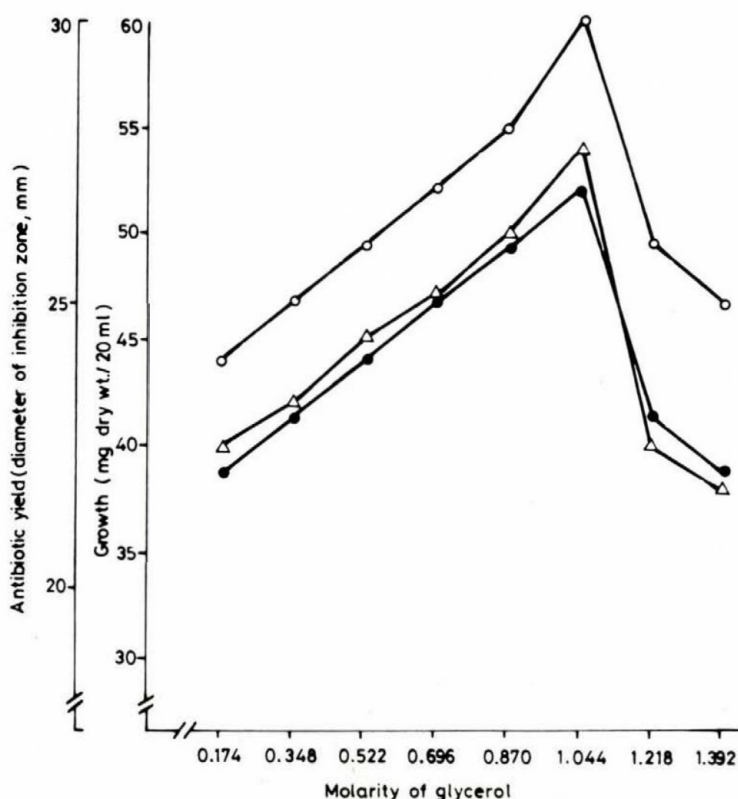


Fig. 1. Effect of glycerol on growth and antibiotic yield by *S. antibioticus* Sr_{15.4}.
 ▲—▲ growth; ○—○ inhibition of *E. coli*; ●—● inhibition of *B. subtilis*

The antibiotic compound is a low melting creamy white solid, highly soluble in chloroform and methanol, aliphatic in nature. The compound showed $\lambda_{\max}$ in chloroform 250.8 nm in the UV-region. Mass spectrum of the compound indicated that molecular weight is 406. The IR absorption spectra envisaged that the compound contained alcoholic -OH and carbonyl groups and absence of absorption bands at 1600cm^{-1} and 1500cm^{-1} in the IR region indicated its non-aromatic nature.

Discussion

It has been reported that several *Streptomyces* species like *S. birdsislandi* [12], *S. faecalis* spp. *liquefaciens* [13] and *S. lydicus* LL-E 19020 [14] are able to produce antibacterial antibiotics extracellularly. *S. antibioticus* Sr_{15.4} [9] an addition to that list.

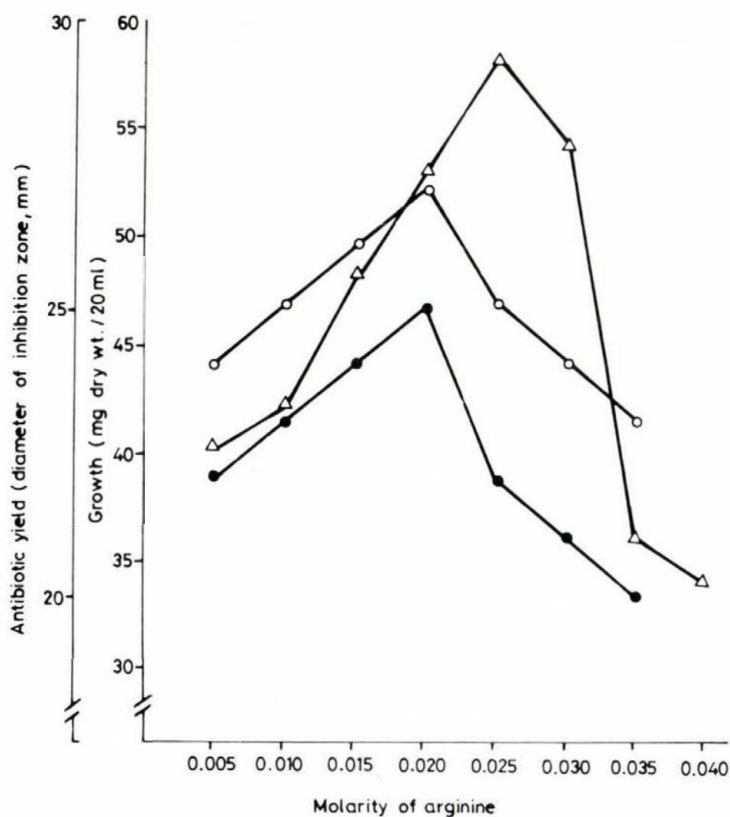


Fig. 2. Effect of different molarity of arginine on growth and antibiotic yield by *S. antibioticus* Sr_{15.4}. For explanation see Fig. 1

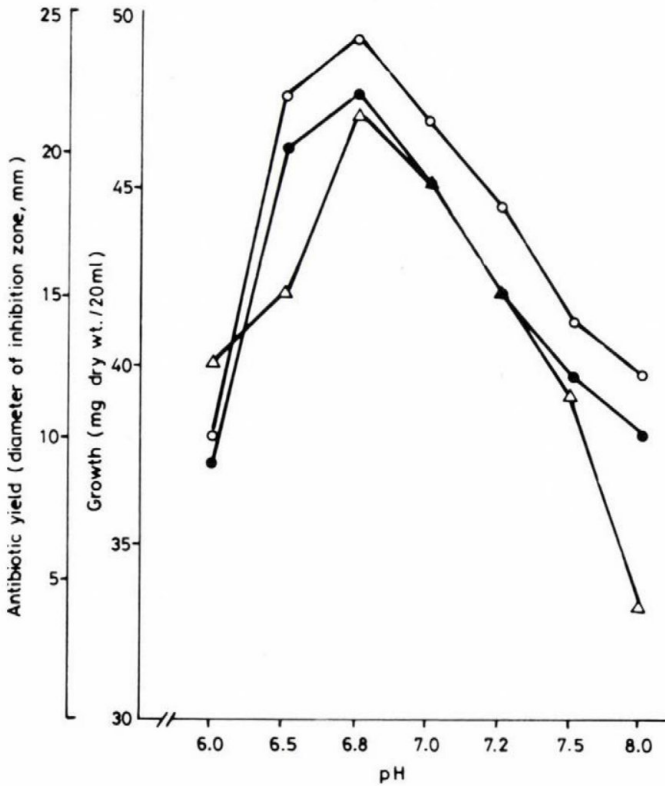


Fig. 3. Effect of pH on growth and antibiotic yield by *S. antibioticus* Sr_{15.4}. For explanation see Fig. 1

Critical examination of the constituents of thirteen different media reveals that the two media are suitable for antibiotic production by the isolate Sr_{15.4}. Soya bean meal and Arginine Glycerol Salt (AGS) broth media have only one common ingredient viz. glycerol (20.0 and 12.5 g/l respectively). However, the lower antibiotic yield in Glycerol Asparagine medium may be due to excess amount of glycerol (35 g/l) in the medium.

In most of the referred cases glucose is the main carbon source [15–19]. But in this strain of *S. antibioticus* Sr_{15.4} glycerol is utilized best and the highest amount of antibiotic production occurs at 1.044 molar concentration, though with glucose the production is not significantly less.

It is reported in some strains of *Streptomyces* that peptone is the best source of nitrogen for antibiotic yield [16, 19] and some strains can also utilize sodium nitrate as the best nitrogen source [18]. *S. antibioticus* Sr_{15.4} enhanced its antibiotic yield when grown in arginine at 0.020 molar level.

S. antibioticus Sr15.4 can withstand a wide range of pH, i.e., 6.5–7.5. But maximum production of antibiotic was noticed when grown at pH 6.8 which is near neutrality. The available reports also suggest that in antibiotic production of *Streptomyces* is the most active in the range of pH 6.0–7.2 [15, 17, 19, 20].

The antibacterial activity of the culture fluid is at this moment not high which may be either due to a low biological activity or to low production. It is worthy to note that *S. antibioticus* Sr_{15.4} can withstand the changed cultural conditions very easily and requires low amount of different ingredients, hence minimum cost. Furthermore, its optimum pH is also near neutrality. Simultaneously it is also clear that the strain has the potentiality to increase the amount of antibiotic which is hereupto more than one and half-times.

Acknowledgements. The authors are thankful to the Head of the Department for her co-operation and to the University Grants Commission for awarding fellowship and financial assistance. We are also thankful to all other colleagues who extended their helping hands to complete the work successfully.

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IN VITRO SUPPRESSION OF ANTI-TSH RECEPTOR ANTIBODY BY AUTOLOGOUS ANTI-IDIOTYPIC ANTIBODY IN PATIENTS WITH GRAVES' DISEASE

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(Received January 12, 1994)

(Accepted March 8, 1994)

Regulation of anti-TSH receptor antibody (anti-TSH-R antibody) in Graves' patients ($n=11$) by anti-idiotypic antibody was studied using patients sera before and after one year of Methimazole treatment. Patients sera with high level of anti-TSH-R antibody (110 ± 41.9 U/l) were incubated with pooled control and autologous (with low level or anti-TSH-R antibody negative) sera containing equimolar IgG G. The mixture was centrifuged and the supernatants were tested for anti-TSH-R antibodies (TRAK, Henning). It was found that the autologous sera from patients with remission were able to suppress significantly the titre of anti-TSH-R antibodies ($p<0.001$), whereas the controls were capable of a less remarkable inhibition. $F(ab')_2$ fragments of autologous IgG from remission could also suppress the levels of TSH-R antibodies. It was concluded that anti-anti-TSH-R antibodies in sera of Graves' patients might be, at least in part, responsible for inducing and maintaining remission and suppression of autoantibodies to TSH-R. It is hypothesized that the idiotype system is part of the network of natural autoantibodies and that its perturbation may give rise to pathogenetic antibodies. On the basis of this observation the autologous sera could have therapeutical implication in an accelerated remission of hyperthyroidism in Graves' disease.

Recently, there is accumulating evidence for idiotypic-anti-idiotypic regulation in autoimmune diseases [1, 2]. The emergence of autoimmunity in Graves' disease may result from an up-regulated expansion of autoreactive clone(s) to TSH-receptor (TSH-R) [2, 3]. Anti-TSH-R antibodies have been shown to be anti-TSH anti-idiotypic antibodies [4]. Several observations confirm the time-dependent decrease of anti-TSH-R antibodies in remission induced by Methimazole [5, 6, 7]. It was assumed that Methimazole-induced recovery of thyrotoxicosis might be elicited by production of anti-idiotypic antibodies to TSH-R antibodies.

This was prompted us to gain more insight into mechanism of idiotypic-anti-idiotypic regulation and the process of Methimazole-induced recovery followed by disappearance of anti-TSH-R antibodies.

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Materials and methods

Patients. Eleven untreated patients (9 females, 2 males with hyperthyroid Graves' disease aged 23–61 years, average 41.1 years) were studied. All patients exhibited characteristic features of Graves' hyperthyroidism and five had infiltrative ophthalmopathy as well. Thyrotoxicosis was confirmed by a high level of serum triiodothyronine (TT₃ 6.7±1.9, normal range 0.52–2.5 nmol/l (Amerlite), thyroxine (TT₄ 229±38 nmol/l, normal range 65–160 nmol/l) (Amerlite) and suppressed TSH (0.08 mU/l, normal range 0.15–3.2 mU/l) (Amerlite monoclonal assay). All patients were treated with Methimazole for 12 months (average dose 15 mg/day) and steroid therapy (starting dose of Prednisolone 55 mg/day) was added in five patients with ophthalmopathy. Serum anti-TSH-R-, anti-human-thyroglobulin (anti-hTG), anti-thyroid peroxidase (anti-TPO) antibodies were measured at presentation and at 2–3 month intervals until treatment was discontinued.

Methods. Anti-TSH-R antibodies were determined by radioreceptor standard assay (TRAK, Henning, Berleim) and expressed in U/l. Values above 12.0 U/l were considered positive.

Anti-human thyroglobulin (anti-hTg) and anti-thyroid peroxidase (anti-TPO) antibodies were measured by ELISA. Preparation of human thyroglobulin and TPO/microsomal antigens were made and purified from the saline extracts of toxic goitres by ammonium sulphate fractionation and sepharose 6B chromatography according Hamada et al. [8]. Human TPO was obtained from the 70 000 g pellet of thyroid homogenate after solubilization with deoxycholate in the 100 000 g supernatant fraction using the method of Taurog et al. [9] and Kohno et al. [10]. Indirect ELISA was performed by modification of Vollers's method [11]. Enzy-plate (Propylen GM, Hungary) were coated with 100 µl of hTG or TPO fractions (each 10 µg/ml) by overnight incubation at 4 °C. Aliquots of 100 µl sera (diluted 1:100) were added to the wells and incubated for 2 h at room temperature. Goat anti-human IgG antibodies conjugated with horseradish peroxidase (Human, Hungary) were chosen for detection of antibody binding (dilution 1:1000). o-Phenyldiamine was used as substrate. Optical density was measured by ELISA reader at 492 (Eurogenetics). The results were given as an index of the OD ration of triplicates of patients' sample to the mean OD of control samples × 100. Results were considered to be positive above 200.

Detection of anti-TSH antibodies. ¹²⁵I-TSH (Izinta, Budapest) was incubated with 0.01 ml of test serum in a total volume of 1 ml containing Tris/NaCl plus 1% bovine serum albumin (assay buffer). After overnight incubation at 4 °C, 0.05 ml of goat anti-human IgG (ATAB Scarborough, USA) was added and incubation continued overnight at 4 °C. Then the bound and free fractions were separated by centrifugation at 2000 g for 30 min. The pellets were washed twice with assay buffer to reduce non-specific binding. The level of binding (B/T%) of control pooled sera from healthy donors was 2.1±0.33.

Preparation of IgG. IgGs were isolated and purified from sera by affinity chromatography using ImmunoPure(G) IgG Purification kit (Pierce 44441, USA). Concentration of IgGs were measured by laser nephelometric assay (Hyland nephelometer).

Preparation of F(ab')₂ fragments of IgG. F(ab')₂ fragments were prepared by digestion of purified IgGs with pepsin digestion using ImmunoPure F(ab')₂ preparation kit (Pierce 44888, USA). The purity of the F(ab')₂ preparation was tested by Ouchterlony double immunediffusion using anti-human IgG F(ab')₂ (Heintel, Austria). Suppression of anti-TSH-R antibodies was determined by autologous sera after 1 year Methimazole therapy. Aliquots of 100 µl patients sera or purified IgGs were incubated with pooled control or autologous sera or purified IgGs of equimolar concentrations (1.0 g/l) at 37 °C for 1 h, then at 4 °C for 18 h. The mixture was centrifuged at 10 000 g and the supernatants were tested for anti-TSH-R antibodies. A similar procedure was carded out by purified F(ab')₂ fragments of homologous and autologous IgGs. The suppression was calculated from the formula:

$$1 - \frac{\text{titre of anti-TSH-R antibody in presence of serum or IgG} \times 100}{\text{titre of anti-TSH-R antibody in absence of serum or IgG}}$$

Results

Antibodies to TSH-R, TPO-, hTG, TSH. All patients were positive for anti-TSH-R antibodies before Methimazole treatment. An apparent decrease in thyrotropin binding inhibitory immunoglobulin (TBII) was observed during Methimazole therapy, however after 12 months it remained elevated in six patients (Fig. 1). Levels of anti-TPO antibodies were slightly elevated in six sera and there was a gradual fall in their titre. Similarly, the titres of anti-hTG antibodies were above the control values in three patients and at the end of treatment no considerable change was observed. Anti-TSH antibodies were not detected in any patients sera.

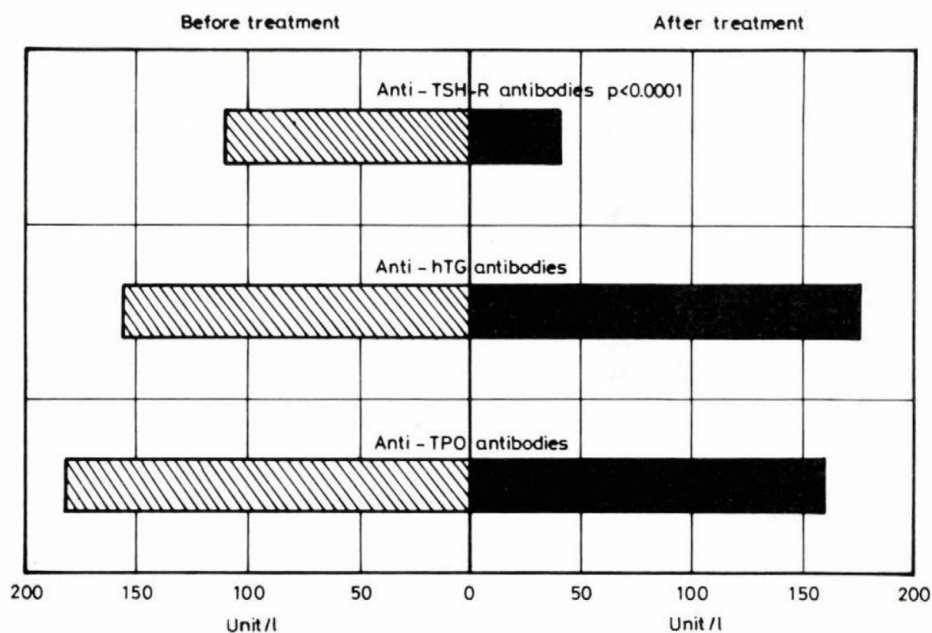


Fig. 1. Anti-thyroid antibodies in sera of patients with Graves' disease. Anti-TSH-R, anti-hTG-, anti-TPO antibodies were measured in sera as written in Materials and methods. The changes in titre of these antibodies were evaluated after 1 year Methimazole treatment. Titre of anti-TSH-R antibodies was significantly decreased ($p < 0.0006$) after Methimazole treatment in comparison to untreated values. Levels of anti-hTG and anti-TPO antibodies were not different before and after treatment

Suppression of anti-TSH-R antibodies by homologous (pooled) and autologous sera. To test whether autologous recovery and homologous sera contain inhibitory component(s) we determined their effect on level of anti-TSH-R antibodies. Both homologous and autologous (after 12 month Methimazole treatment) sera were able to decrease the level of anti-TSH-R antibodies. Surprisingly, the percentage of inhibition proved to be higher in the presence of autologous sera containing equimolar IgGs than that of homologous sera (Fig. 2). We examined the antibodies to TPO as well as hTG in the presence of autologous and homologous sera and observed an inhibitory effect, however, it was less impressive because the levels of anti-TPO antibodies were slightly elevated.

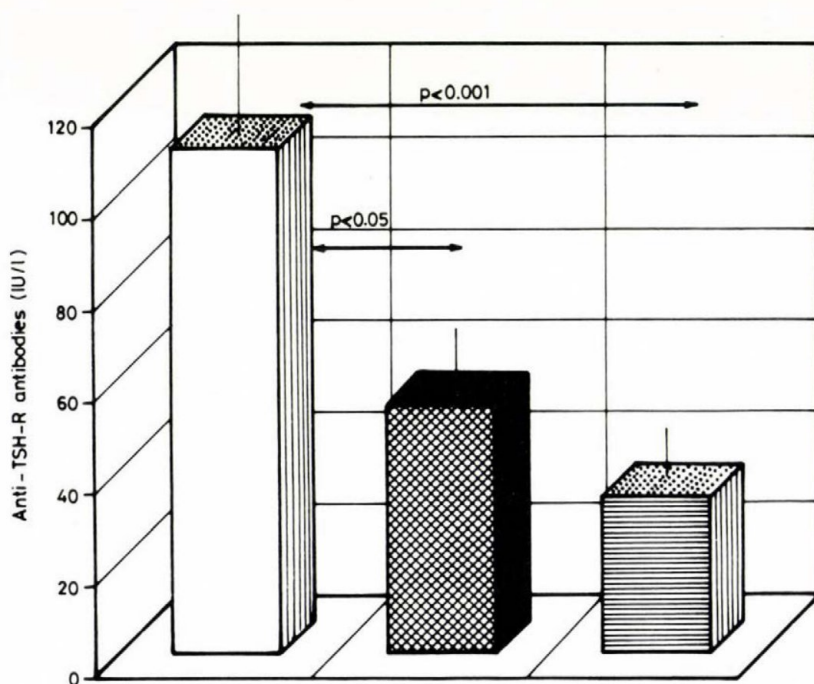


Fig. 2. In vitro suppression of anti-TSH-R antibodies (open bar) by homologous (shaded bar) and autologous (hatched bar) sera with equimolar IgGs (1.0 g/l). After incubation and centrifugation the titre of anti-TSH-R antibodies was determined in the supernatants. The autologous sera significantly decreased the titre of anti-TSH-R antibodies as compared to that of homologous sera ($p < 0.001$ vs. 0.05)

Study of suppressive effect of autologous IgGs and $F(ab')_2$ on anti-TSH-R antibodies. To study the possible suppressive role of immunoglobulin fraction, IgGs were prepared and purified from autologous and homologous sera. Both autologous and homologous IgGs suppressed the anti-TSH-R antibodies. In addition, the autologous

IgGs showed a higher dose-dependent decrease in titre of antibodies to TSH-R than that of equivalent amount of homologous IgGs (Fig. 3). Thereafter, $F(ab')_2$ fragments were purified from four patients' and control IgGs. The $F(ab')_2$ fragments of autologous IgGs were able to suppress the level of anti-TSH-R antibodies (Table I).

Table I

In vitro suppression of anti-TSH-R antibodies (TBII) of untreated patients by recovery $F(ab')_2$ fragments. $F(ab')_2$ fragments were prepared by digestion of pepsin using ImmunoPure $F(ab')_2$ preparation kit

Experiment	1	2	3	4	Mean
TSH-R antibodies	118	212	98	88	129
TSH-R antibodies by autologous	23	41	28	21	28.5
IgG $F(ab')_2$					
Suppression (%)	80.4	80.2	71.4	76.2	77
TSH-R antibodies by homologous	42	77	40	37	54.5
IgG $F(ab')_2$					
Suppression (%)	64.1	63.3	59.1	57.8	60

Discussion

Graves' disease is characterized by increased levels of anti-TSH-R antibodies which are able to stimulate function of thyroid gland [2, 3]. The levels of anti-TSH-R antibodies have been found to be gradually decreased during therapy with Methimazole [5, 6, 14]. The mechanism of this decrease has not yet been clarified. Both direct immunosuppressive effects in target organ (free oxygen radical scavenging capacity) and indirect effects on autoantigen presentation by reducing HLA-DR expression on thyrocytes have been published [12–17]. In this report we have observed a decrease in anti-TSH-R antibody titres after 1 year treatment with Methimazole and examined the effect of recovery autologous sera on pretreatment anti-TSH-R antibodies. We have documented that autologous sera of patients from remission possess a higher reducing capacity on titre of anti-TSH-R antibodies than that of homologous sera. This phenomenon can be explained by possible presence of anti-idiotypic antibodies which are able to bind anti-TSH-R antibodies.

This possibility was confirmed by our study with purified IgGs and $F(ab')_2$ fragments. The similar favourable effect of autologous anti-idiotypic antibodies prepared from sera in remission on prerecovery autoantibodies have been found in other autoimmune disorders including SLE, myasthenia gravis, rheumatoid arthritis as well [18–21]. Sera from healthy individuals and IgGs should contain anti-idiotypic antibodies to anti-TSH-R antibodies at much lower concentrations than that of post-

recovery IgGs. This finding indicates the high capacity of sera and IgGs from recovery for reducing anti-TSH-R antibodies.

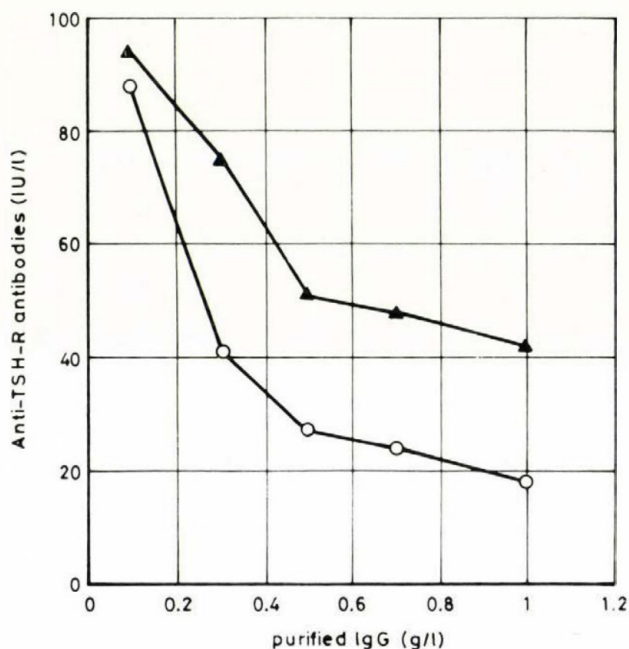


Fig. 3. In vitro dose-dependent suppression of anti-TSH-R antibodies by recovery autologous (open circle) and homologous (triangle) IgGs. IgGs were prepared from the recovery serum of patient 5 and the sera of healthy donors (pooled sera) by ImmunoPure(G) IgG purification kit. The purified IgGs were added to the patients IgG with various mounts. After incubation and centrifugation the anti-TSH-R antibodies were tested in the supernatants

We believe that the enhanced level of anti-TSH-R anti-idiotypic antibodies in recovery sera is responsible for neutralizing the TSH-R antibodies at larger extent than that of healthy individuals' sera. Nevertheless, these experiments provide further evidence for an idiotype connection between antibodies to TSH-R. It is hypothesized that this idiotype system is part of the network of natural autoantibodies and that its perturbation may give rise to pathogenetic antibodies. In addition, this observation seems to be important from therapeutical viewpoint since by infusion of autologous post-recovery sera containing anti-idiotypic antibodies to TSH-R antibodies of high level may induce the recovery of Graves' hyperthyroidism.

Acknowledgement. This work was supported by the Hungarian Research Fund (OTKA 5185).

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EFFECTS OF SOME HEAVY METALS ON GROWTH, PIGMENT AND ANTIBIOTIC PRODUCTION BY *STREPTOMYCES GALBUS*

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(Received February 14, 1994)

(Accepted April 13, 1994)

A chromogenic strain of *Streptomyces galbus* SME-13 producing an antifungal antibiotic was used to study the effects of some heavy metals on growth, melanoid pigment and antibiotic production in culture. Mercury and nickel were highly toxic followed by cadmium while cobalt and chromium appeared to be less toxic. All the metals inhibited growth, pigment and antibiotic production with widely variable IC_{50} values. Cadmium and chromium at lower concentrations unusually stimulated growth and enhanced pigment production in the strain.

Heavy metals include a wide array of elements with diverse chemical and biochemical properties. Many of them (e.g., Cu, Mn, Fe and Zn) are essential for living organisms at a very low concentrations (micronutrients), while others like Hg, Pb and Cd do not have any essential biological functions and could be toxic to them. It is well known that the industrial and domestic activities are continuously emitting large quantities of heavy metal pollutants, both organic and inorganic in the environment. Toxicity of these accumulated heavy metal pollutants to the living system is well documented and results in cell death or impairment of growth and metabolism. The biochemical and physiological mechanisms of toxicity of heavy metals on microorganisms have been reviewed extensively by various workers [1–5] and include denaturation of proteins including enzymes, binding and disruption of cell membrane and acting as antimetabolites towards essential nutrients. The degree of toxicity of heavy metals to microbes is also markedly influenced by biotic and abiotic environmental factors [5–8]. Likewise, production of secondary metabolites by microorganism is also known to be affected by the presence or absence of heavy metals as they may be responsible for activation of some of the biosynthetic pathways [9, 10].

Streptomyces galbus SME-13 is known to produce a new antifungal antibiotic and also to release a melanoid pigment in the culture medium, the intensity of which increases with the period of incubation. Regulatory effects of some microelements like Cu, Fe, Mn and Zn on the growth and antibiotic production by *S. galbus* were also reported [11, 12]. Effects of heavy metals on secondary metabolism of streptomycetes

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particularly on the antibiotic production has not been studied extensively. Growth and actinorhodin production by *S. coelicolor* as influenced by heavy metals has, however, been studied as a model system to examine the effects of metals on growth and polyketide synthesis in a streptomycete [13].

This study gives data on the influence of some heavy metals on the growth, pigment and antifungal antibiotic production by *S. galbus*.

Materials and methods

Bacterial strain. *S. galbus* 5ME-13 was maintained on glucose asparagine agar slants containing (g litre⁻¹) asparagine (BDH, England), 0.5; K₂HPO₄, 0.5; glucose, 10 and agar, 20 (pH 6.8). Spore suspensions were prepared in sterile 0.1% Tween 80 (Hi Media, India) solution from 14 days old slant cultures grown at 30 °C.

Culture medium. To study the effects of heavy metals on the growth and antibiotic production, the organism was grown in Lindenbein synthetic medium that contained the following (g litre⁻¹): glycerol, 30; K₂HPO₄, 1; MgSO₄·7H₂O, 0.5; KCl, 0.5; FeSO₄·7H₂O, 0.1; NaNO₃, 2.0 (pH adjusted to 7.2). All the chemicals used in this experiment were of analytical reagent grade and unless otherwise mentioned were obtained from E. Merck, India. The heavy metals were used as chloride salts, HgCl₂; NiCl₂·6H₂O (B.D.H., India); CoCl₂·6H₂O; CrCl₃; CdCl₂·H₂O (Sigma). To 18 ml of medium per 100 ml flask 1 ml of previously sterilized metal salt solution was added to get the desired concentrations. Each flask was then inoculated with 1 ml of homogeneous spore suspension (OD=0.2) and incubated at 30 °C for 8 days.

Measurements of growth, antibiotic and pigment. Growth of the producer strain was measured as dry weight of the mycelium. Antibiotic titre of the culture filtrate was determined by agar-cup assay using *Curvularia lunata* as the test organism. The assay plates were prepared by mixing 0.5 ml of freshly prepared spore suspension of the test organism with 20 ml of Potato dextrose agar. Agar cups (8 mm in diameter) were filled with 0.1 ml of culture filtrate in triplicate and the plates were incubated at 30 °C for 2 days. Intensity of the melanoid pigment in the culture filtrate was determined by measuring the optical density at 540 nm with AD 101 Grating Spectrophotometer using uninoculated broth as the control. Values for biomass, antibiotic and melanoid pigment production in metal-treated cultures were expressed as a percentage of those obtained in untreated control which were taken as 100%.

Results

Effects of five different heavy metals viz., mercury, nickel, cadmium, cobalt and chromium on growth, melanoid pigment and antibiotic production by *S. galbus* are shown in Figs 1-5.

Mercury was found to be extremely toxic and inhibited growth, antibiotic and pigment production. A total inhibition of all three processes were recorded at 0.5 µg ml⁻¹ (Fig. 1).

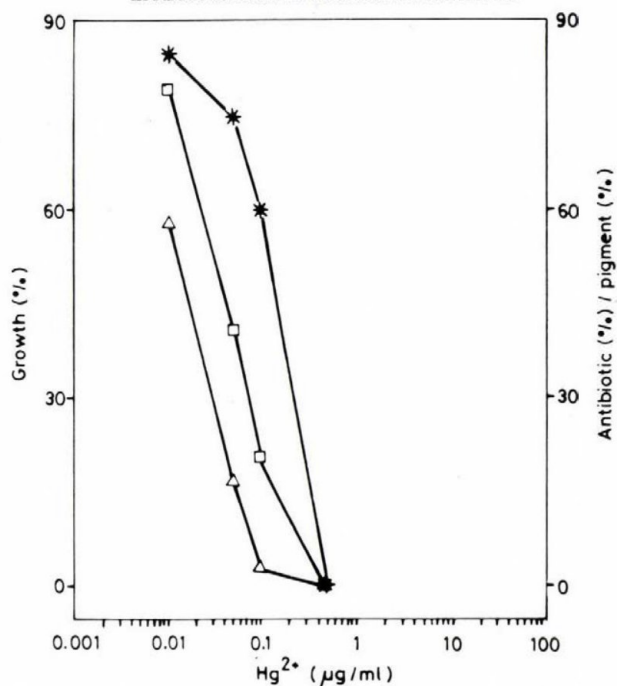


Fig. 1. Effect of mercury on growth ($\square$), pigment (Δ) and antibiotic ($*$) production by *S. galbus*. 18 ml of Lindenbein synthetic medium/100 ml flask supplemented with 1 ml of salt solution and inoculated with 1 ml of spore suspension; Incubation: 8 days at 30 °C

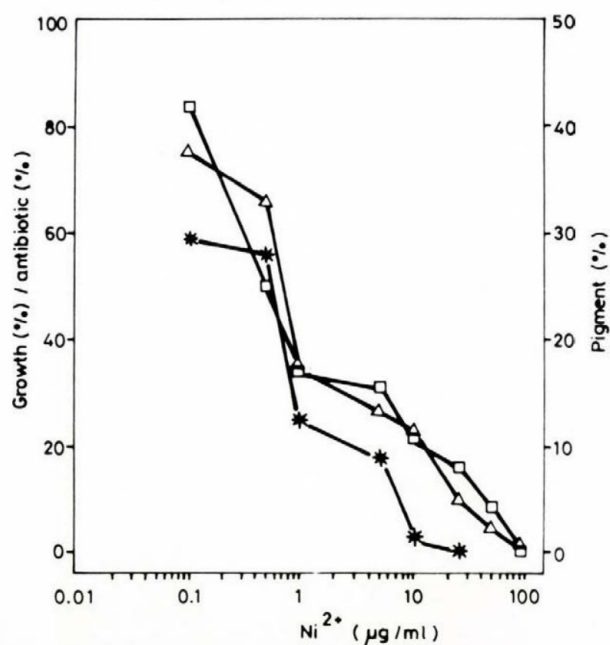


Fig. 2. Effect of nickel on growth ($\square$), pigment (Δ) and antibiotic ($*$) production by *S. galbus*.
Explanation as in Fig. 1

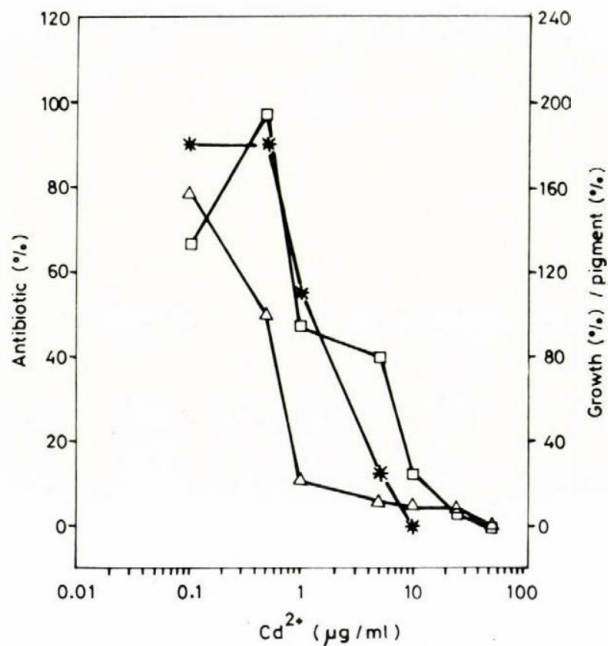


Fig. 3. Effect of cadmium on growth ($\square$), pigment (Δ) and antibiotic ($*$) production by *S. galbus*.

Explanation as in Fig. 1

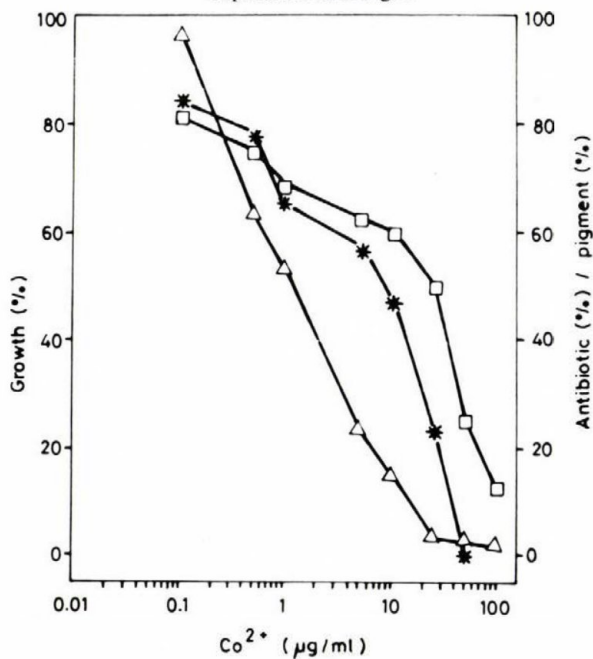


Fig. 4. Effect of cobalt on growth ($\square$), pigment (Δ) and antibiotic ($*$) production by *S. galbus*.

Explanation as in Fig. 1

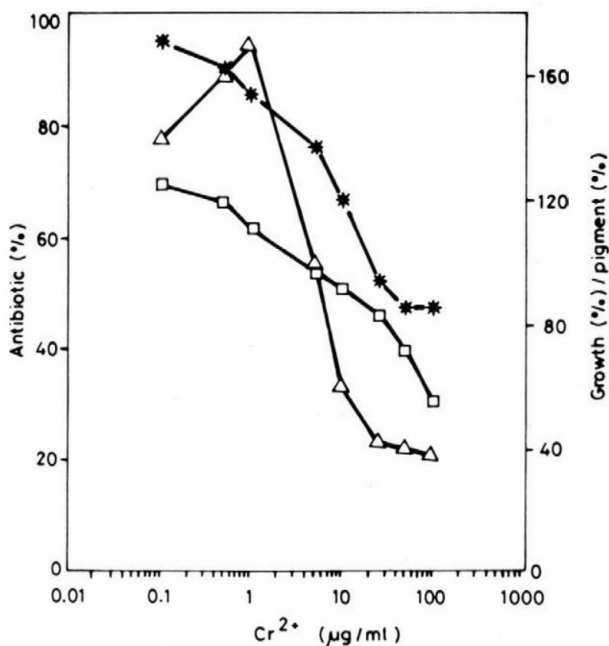


Fig. 5. Effect of chromium on growth (□), pigment (Δ) and antibiotic (*) production by *S. galbus*.
Explanation as in Fig. 1

Nickel also adversely affected the three parameters tested. Growth, pigment and antibiotic production decreased in a more or less regular fashion with increasing concentrations of nickel in the medium. Antibiotic production was totally inhibited at $25 \mu\text{g ml}^{-1}$ while, total inhibition of growth and melanoid pigment was recorded at $100 \mu\text{g ml}^{-1}$ (Fig. 2).

Cadmium showed a complex effect on growth and secondary metabolism of *S. galbus*. Antibiotic production was not significantly affected by lower concentrations ($0.5 \mu\text{g ml}^{-1}$) of cadmium while at higher concentrations the inhibition was very sharp showing a total inhibition at $10 \mu\text{g ml}^{-1}$. Growth and pigment production on the other hand was favoured at lower concentrations. The final biomass yield and pigment production was increased to almost 195% and 157% at $0.5 \mu\text{g ml}^{-1}$ and $0.1 \mu\text{g ml}^{-1}$ respectively followed by a decline at higher concentrations (Fig. 3).

Pigment production declined sharply upto $25 \mu\text{g ml}^{-1}$ of cobalt although a trace amount of pigment production was found even upto $100 \mu\text{g ml}^{-1}$. Growth and antibiotic production showed almost a similar pattern of inhibition with increasing concentrations of cobalt in the medium. Antibiotic production was totally inhibited at $50 \mu\text{g ml}^{-1}$ while, growth was not completely inhibited at the highest concentration ($100 \mu\text{g ml}^{-1}$) tested (Fig. 4).

Like cadmium, chromium also favoured growth and pigment production at lower concentrations. Pigment production was increased almost to 170% at $1 \mu\text{g ml}^{-1}$ and thereafter declined sharply at higher concentrations while biomass production as high as 125% was recorded at the lowest concentration ($0.1 \mu\text{g ml}^{-1}$) but declined gradually with increasing concentration. Antibiotic production, on the other hand, was regularly inhibited from the lowest concentration ($0.1 \mu\text{g ml}^{-1}$) of chromium. None of the three processes, however, were totally inhibited at highest concentrations of chromium (Fig. 5).

Discussion

The toxic effects of heavy metals on growth and different cellular metabolic activities are a matter of concern. The effects of metals in terms of the concentration required to reduce the yield of biomass, antibiotic and pigment to 50% of that of the untreated control are summarized in Table I. Based on their toxic effects on *S. galbus*, the heavy metals tested could be segregated into three categories.

Mercury and nickel constituted the first category and are most toxic. Similar toxic effects of mercury and nickel were also demonstrated on growth and actinorhodin production by *S. coelicolor* [13]. Claridge et al. [14] on the other hand, reported a stimulatory effect of nickel on coumermycin A₁ production by *S. rishiriensis*. The observed effect were presumed to be due to contamination of cobalt (0.4%) in the nickel chloride used.

Table I

*IC*₅₀ values of metals affecting growth, antibiotic and pigment production in *S. galbus*

Metal	Growth	Antibiotic	Pigment
Hg	0.04	0.11	0.015
Ni	0.5	0.6	<0.1
Cd	8.0	1.1	0.8
Co	30.0	9.0	1.12
Cr	>100.0	32.0	16.0

Each value represents the average of triplicate tests

The second category is represented by cadmium and occupies an intermediate position in terms of toxicity. The *IC*₅₀ values of cadmium for all three processes were higher than Hg and Ni. Earlier reports have clearly indicated that *Streptomyces* species are very sensitive to cadmium with regard to growth, sporulation and antibiotic production [13, 15], although Babich and Stotzky [16] reported that actinomycetes as a

group are more tolerant to cadmium than eubacteria. The enhancement of growth and pigment production in *S. galbus* at lower concentrations of cadmium during the present study therefore represent a deviation from that of the earlier observations.

The third category represented by cobalt and chromium had high IC_{50} values and therefore appeared to be less toxic to *S. galbus*. Contrary to the inhibitory effect of cobalt on secondary metabolite production by *S. galbus*, Claridge et al. [14] observed that addition of as little as $0.01 \mu\text{g ml}^{-1}$ of cobalt, resulted in the production of over 93% coumermycin A_1 by *S. rishiriensis* compared to 40–75% production in the control. The enhancement of growth and pigment production by *S. galbus* as influenced by the lower concentrations of chromium during the present study was comparable with that of cadmium. These promotive response of cadmium and chromium appeared to be unusual in nature and deserves special attention.

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ALTERED HUMORAL IMMUNITY AGAINST CYTOMEGALOVIRUS AND EPSTEIN-BARR VIRUS WITHOUT DETECTABLE VIRUS ANTIGENS AND VIRUS-DNA IN THE GLOMERULI OF PATIENTS WITH IGA NEPHROPATHY IN REMISSION PHASE

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(Received March 3, 1994)

(Accepted May 10, 1994)

Herpes viruses, such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV), very often occur in the nasopharynx. A pathogenetic role of these viruses in immunoglobulin A nephropathy (IgA NP) is a challenging hypothesis, since upper respiratory tract infections are frequently and closely related in time to the acute episodes of IgA NP. However, conflicting reports have been published in this field. We compared the IgA and IgG antibody (AB) titres against cytomegalovirus (CMV) in sera of 41 IgANP patients with 80 healthy controls. The prevalence rates of CMV-IgA and CMV-IgG AB were significantly higher in patients with IgA NP than in healthy controls (CMV-IgA, titre ≥ 8 , $p < 0.001$; CMV-IgG, titre ≥ 16 , $p < 0.05$). There was also a significant difference in the concentration of CMV-IgA AB between IgANP patients and controls (IgA NP: 0.34 ± 0.66 , healthy controls: 0.06 ± 0.33 , mean $\pm$ SD, $p < 0.002$), but not in the concentration of CMV-IgG AB between NP patients and controls. While examining 60 patients with IgA NP and 75 healthy controls a significantly elevated prevalence rate of IgA AB against EBV capsid antigen (EBV-VCA) was also detected in the sera of IgA NP patients vs controls (titre ≥ 16 ; $p < 0.05$). There was no significant difference in the prevalence of IgG AB to EBV-VCA between patients with IgA NP and healthy controls, except at titre = 1024 ($p < 0.05$). At a follow-up, CMV-IgA persisted in 4 of 5, and EBV-VCA-IgA in 8 of 12 seropositive patients with IgA NP. We could not detect virus antigens or virus deoxyribonucleic acid (DNA) in the glomeruli of NP patients

*Professor Israel Sarov died suddenly. The authors would like to devote this paper to his memory.

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either with immunohistology using a monoclonal antibody or with the DNA in situ hybridization technique. Our examinations support the hypothesis that most patients with IgA NP have altered humoral immunity with a long-acting IgA response to CMV and EBV antigens. Furthermore, it is concluded that patients with IgA NP do not have a significant presence of CMV antigens or CMV-DNA and EBV-DNA in their glomeruli suggesting that the antigens tested and the whole viruses are probably not present in the glomerular pathologic changes.

Substantial evidence now points to the important role of IgA IC in the pathogenesis of IgA NP [1-9]. The presence of circulating IC is intermittent and closely related in time to mucosal infections of the respiratory, gastrointestinal or urinary tracts followed shortly by episodes of acute exacerbations of the disease, accompanied with macroscopic haematuria. It is suggested that, being deposited in the glomeruli around the time of the mucosal infection, IC may trigger the clinical activity of IgA NP, sometimes with acute worsening of the disease. Such rapid events, however, suggest the presence of preformed antibodies against antigens taking part in the process.

Herpes viruses very often occur in the nasopharynx causing upper respiratory tract infections. Therefore in our previous investigations using indirect immunofluorescence and complement fixation to study circulating antibodies to EBV, HSV and CMV antigens we assumed a pathogenetic role for these viruses in some patients with IgA NP [10, 11]. Since then, some workers have confirmed and others refused this assumption, mainly in connection with CMV [12-23].

In this study (1) we examined IgA and IgG antibodies against CMV and EBV-VCA by a sensitive immunoperoxidase method. Using follow-up and re-examination of seropositive patients we hoped to obtain data on the persistence of IgA antibodies to CMV and EBV-VCA in IgA NP patients. (2) To obtain data on the direct pathogenetic role of CMV and EBV in some patients with IgANP, we studied the glomerular presence of these viruses in renal biopsies using different methods (indirect immunoperoxidase and in situ DNA hybridization).

Material and methods

I. Serological examinations

1. CMV studies

Patients. (a) Forty-one patients with IgA NP (32 men and 9 women; 28 to 71 years of age), (b) 80 healthy controls (52 men and 28 women; 16 to 67 years of age). All the IgA NP patients had persistent mild proteinuria and/or microscopic haematuria but none of them had macrohaematuria at the time of examination.

Method. CMV IgA and IgG antibodies in the sera of patients and controls were studied by an indirect immunoperoxidase method as previously described [24], using human embryonic fibroblast cell suspension infected with CMV (AD 169 strain) as antigen. Each test included known positive and negative sera. Sera were tested from dilution 1:8 in the IgA antibody assays and 1:16 in the IgG antibody assays, titration being performed to the end points. IgA titres ≥ 8 and IgG titres ≥ 16 were considered positive.

2. EBV studies

Patients. (a) Sixty patients with IgA NP (47 men and 13 women; age range 18 to 71 years), (b) 75 healthy controls (59 men and 16 women; age range 17 to 60 years).

Method. Sera were tested for specific IgA and IgG anti-EBV VCA by indirect immunoperoxidase assay (IPAzyme EBV/VCA, Savyon Diagnostics Ltd, Beer-Sheva, Israel) utilizing P3HR-1 Burkitt's lymphoma cell line as antigen. The sera were tested from dilution 1:8 for IgA and 1:64 for IgG. When 4-chloro-1-naphtol was used as substrate it gave IgA and IgG antibody titres twice as high as those obtained previously with benzidine [25].

II. Morphological examinations

Material. Cryostat sections of kidney biopsies obtained from 18 patients with IgA NP, were studied by the PAP method for the presence of CMV antigen. In addition, paraffin sections of kidney biopsies obtained from 19 patients with IgA NP, were examined using the DNA in situ hybridization method for the presence of CMV and EBV antigens.

Methods. CMV antigen examinations on cryostat sections. In preliminary experiments the reactivity of two monoclonal antibodies against CMV early and late antigens (Biotechnika, Budapest, Hungary) was investigated on fixed fibroblasts infected with CMV (AD 169 strain) used in our serological examinations. Only the antibody against CMV early antigens showed consistently positive staining, and, therefore, only this antibody was used for staining the cryostat sections of renal biopsies, which was followed by staining with goat antibody against mice immunoglobulins and mice PAP complex (Heintel, Vienna, Austria).

DNA probe based assay on paraffin sections for CMV [26] and EBV examinations. Kidney biopsies were studied for the presence of CMV-DNA and EBV-DNA by in situ hybridization method using CMV and EBV Pathogene Kits (Enzo Diagnostics, New York, USA). The assay was performed according to the manufacturer's instructions for formalin-fixed tissue. Briefly, paraffin embedded renal biopsy sections were deparaffinized by xylene (3×3 min) and dehydrated by graded alcohol (50%, 70%, 96%, absolut methanole, 4×5 min). After dehydration the sections were digested by proteinase K at 37 °C for 15 min. To abolish any peroxidase activity, the slides were incubated in hydrogen peroxide solution at 37 °C for 10 min. After dehydration the slides were hybridized using DNA Probe Reagent at 92 °C for 5 min and treated with Post Hybridization Reagent, containing biotinylated CMV-DNA or EBV-DNA, at room temperature for 15 min. For the detection of biotinylated DNA, the sections were incubated with avidin-biotinylated horseradish peroxidase complex at room temperature for 12–15 min. Peroxidase reactivity was demonstrated with aminoethylcarbazole as substrate. As positive controls the same slides were used as in the serological examinations of CMV and EBV antibodies in addition to the control slides from the CMV and EBV-DNA probe Kits. Additionally, paraffin sections from an autopsy case with characteristic cytoplasmic inclusions in cytomegalic cells within renal proximal tubular epithelial cells were employed.

Serological follow-up studies. At 12 to 27 months after the first examination, we re-examined sera of CMV and/or EBV-IgA seropositive patients to detect the assumed persistence of IgA antibodies. For CMV antibodies we re-examined 5 CMV-IgA seropositive patients. For EBV antibodies we re-examined 12 IgA NP patients with previous EBV-VCA seropositivity. CMV and/or EBV IgG antibodies were also re-examined in each case along with IgA antibodies. However, we did not have an opportunity to re-examine the healthy controls.

Statistical analysis. The geometric mean titres (GMTS) of IgA and IgG antibodies in healthy individuals and patients were compared using the unpaired t test. The Chi-square test was employed to compare in each group the number of individuals with the IgA or IgG titres to CMV or EBV equal to or greater than the level of positivity of each test. Differences were considered significant when $p < 0.05$.

Results

Serological examination of CMV and EBV antibodies. Table I shows the prevalence rates of CMV IgA antibodies which were significantly higher in IgA NP patients than in healthy controls (titre $\geq$ 8, $p<0.001$). There was also a significant difference in GMT between IgA NP patients and healthy controls ($p<0.002$ data not shown).

Table I

Prevalence of CMV-IgA antibodies in patients with IgA NP and in healthy controls

	No. of patients	≥ 16 (%)	≥ 32 (%)	$= 64$ (%)
IgA NP	41	11 (27)*	2 (5)	1 (3)
Control	80	3 (4)	2 (3)	0

* $p<0.001$ vs control

Table II

Prevalence of EBV-VCA-IgA antibodies in patients with IgA NP and in healthy controls

	No. of patients	≥ 8 (%)	≥ 16 (%)	$= 32$ (%)
IgA NP	60	18 (30)*	5 (9)	2 (4)
Control	75	11 (15)	6 (8)	1 (1)

* $p<0.001$ vs control

The prevalence rates of EBV-IgA antibodies were also significantly higher in IgA NP patients than in healthy controls (titre $\geq$ 16, $p<0.05$, Table II). We detected no significant difference in the GMT of EBV IgA between renal patients and healthy controls (data not shown).

Table III demonstrates the prevalence rates of CMV-IgG antibodies which were significantly higher in IgA NP patients than in healthy controls (titre $\geq$ 16, $p<0.05$), however, no difference in GMT of CMV-IgG antibodies between renal patients and healthy controls was found (data not shown).

Table III*Prevalence of CMV-IgG antibodies in patients with IgA NP and in healthy controls*

	No. of patients	≥ 16 (%)	≥ 32 (%)	≥ 64 (%)	≥ 128 (%)	≥ 256 (%)
IgA NP	41	38 (93)*	29 (73)	19 (48)	8 (20)	0
Control	80	60 (75)	48 (60)	33 (41)	15 (19)	2 (3)

* $p < 0.005$ vs control

There was no statistically significant difference between the prevalence rates and GMTs of EBV-IgG antibodies in IgA NP patients and healthy controls, except at titre=1024 (Table IV).

All the IgA seropositive patients and controls were also IgG seropositive.

Table IV*Prevalence of EBV-VCA-IgG antibodies in patients with IgA NP and in healthy controls*

	No. of patients	≥ 64 (%)	≥ 128 (%)	≥ 256 (%)	≥ 512 (%)	≥ 1024 (%)
IgA NP	60	58 (96)	50 (83)	37 (62)	23 (38)	12 (20)*
Control	75	70 (93)	59 (79)	43 (57)	21 (28)	6 (8)

* $p < 0.005$ vs control

Serological re-examination. Table V summarizes the results of the serological re-examinations showing that CMV-IgA and EBV-IgA persisted in most of the patients with IgA NP.

Table V*Prevalence rate of CMV-IgA and EBV-IgA antibodies at serological re-examination*

Type of renal disease	Type of antibodies	No. of re-examined patients	Seropositive patients at re-examinations	
			No.	%
IgA NP	CMV-IgA	5	4	80
	EBV-IgA	12	8	66

Morphological examinations. The immunoperoxidase assay and the DNA in situ hybridization technique, detected no CMV antigens, and no CMV-DNA or EBV-DNA in the renal glomeruli of patients with IgA NP.

Discussion

The potential role of viral antigens in the pathogenesis of different IC GNs, e.g. IgA NP, remains a challenging hypothesis. All the more so since some of the viruses tend to persist in the cells of the host, often in latent form, and may cause chronic antigen stimulation with the constant formation of antibodies and, probably, IC.

Since at the onset and in the course of IgA NP, upper respiratory tract infections are frequently followed by periods of acute exacerbation, herpes viruses, mainly EBV (occurring very often in the nasopharynx) have aroused our interest [10–11]. EBV and CMV give rise to a very common chronic, mainly latent infection proved by lifelong persistence of specific IgG viral antibodies. At the same time, IgM and IgA antibodies decline to undetectable levels during convalescence from the primary infection [27–29]. Furthermore, EBV infection results in polyclonal activation of B cells (and/or T cells) and preferentially stimulates the synthesis of IgA [30].

Regarding the kidney, CMV can directly infect renal tubular epithelial cells in cytomegalic inclusion disease of the newborn [31] in vitro mesangial cells [32] and interstitial infiltrating cells in transplanted kidneys [33]. The latter cells, however, may represent blood mononuclear cells in which CMV genome persistence was demonstrated following primary infection [31]. Furthermore, in manifest CMV infection, circulating CMV antigen-antibody IC and their glomerular deposition were also demonstrated in humans [34–35] and in experimental animals [36], but an observation contrary to these was also published [37]. Mainly tubulointerstitial nephritis and rarely GN have been described in patients with apparent EBV mononucleosis. The diagnosis was based mainly on the typical clinical picture and light microscopical examination of the renal biopsies [38–40].

There have been several contradictory reports on the role of CMV in the pathogenesis of IgA NP [10, 11, 13–19, 21–23]. Gregory et al. [15] demonstrated the presence of CMV immunoreactivity in the mesangium of all 31 patients with polyclonal anti-CMV antibodies. Our immunohistological results taken together with the work of Waldo et al. [23] Dueymes et al. [14] and Sato et al. [21] permit the conclusion that the previously reported results [15] are suggestive of the presence of contaminating antibodies to human (mainly fibroblast) antigens in polyclonal CMV antisera. However, our inability to detect CMV antigens in the glomeruli using monoclonal antibodies does not necessarily mean that the particular antigens are absent, since monoclonal antibodies react only with a restricted number of epitopes. These epitopes may be hidden, masked, lost or destroyed in the tissues or might not be expressed among the CMV antigens used for the production of the monoclonal antibodies. However, for the diagnosis of CMV infection, in situ hybridization and the PCR are more sensitive and specific methods, providing direct evidence of viral involvement by identification of a replicating virus in the renal tissue. Other authors and we also failed to detect CMV-DNA with these methods [13, 19, 22]. In a recent

paper, however, Muller et al. [18] using PCR, demonstrated the presence of CMV-DNA in the renal tissue of 10 IgA NP patients. Although the efficiency of these methods is decreased when paraffin embedded tissue is used, as was done in our laboratory, the results of Muller et al. [18] need confirmation based on analysis of a larger number of renal biopsies, since the PCR may detect blood mononuclear cells infected with CMV in renal biopsies [31].

The prevalence rates of CMV and EBV IgA antibodies were significantly higher in our patients than in healthy controls. In contrast with this observation, Waldo et al. [23] examining the ratio of CMV IgG and IgA with RIA did not find any significant difference between IgA NP patients and controls. Beukhof et al. [13] using ELISA detected IgA antibodies to CMV late antigen in 1 out of 29 IgA NP patients. But the prevalence rates and titres of EBV-VCA IgA antibodies were higher also in their IgA NP patients. In addition, Andre et al. [12] found an elevated number of positive patients with anti-EBNA2 (EBV nuclear antigen) antibodies and with anti-EBV-antibodies in IgA NP. However, they did not indicate the isotype of the detected antibodies. The results of our serological examinations are in agreement with some previous observations that IgA NP patients have increased IgA response to different and not only viral antigens acting at mucosal surfaces [41–44].

Although, normally, CMV and EBV IgA antibodies do not appear in the circulation after primary infection, we found that they persisted in most of our re-examined IgA NP patients. Similarly, unusually high frequency and persistence of EBV IgA antibodies have been reported only in NPC and AIDS [45–48]. However, our patients did not suffer from these forms of malignancy and were HIV negative (unpublished observation). Likewise the IgA antibodies persisted in IgA NP patients who were clinically in remission. These data along with a detailed analysis of the function of T and B cells in IgA NP patients and with observations on animal models of IgA NP suggest an abnormal mucosal immunity in this disease [49]. The primary defect may be in the regulation of the immune response to hyperfunction of both suppressor T cells and helper T cells.

Furthermore, our results also support the possibility that the primary defect is in the inadequate suppression of the IgG antibody response to persistent mucosal exposure to environmental antigens [49]. However, our examinations do not support the concept that CMV and EBV IC are directly involved in the pathogenesis of IgA NP.

Acknowledgements. The study was supported by the Scientific Committee of Ben Gurion University, Beer-Sheva, Israel and the Hungarian Research Fund (OTKA and ETT), Hungary

Abbreviations

AB antibody
CMV cytomegalovirus
DNA deoxyribonucleic acid
EBV Epstein-Barr virus
EBNA Epstein-Barr virus nuclear antigen
EBV-EA Epstein-Barr virus early antigen
EBV-VCA Epstein-Barr virus capsid antigen

ELISA enzyme-linked immunosorbent assay
 GMT geometric mean titre
 GN glomerulonephritis
 HSV herpes simplex virus
 IC immunocomplex(es)
 IgA NP IgA nephropathy
 NPC nasopharyngeal carcinoma
 PCP polymerase chain reaction
 RIA radioimmunoassay

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LONGITUDINAL IMMUNOLOGICAL FOLLOW-UP OF HIV INFECTED HAEMOPHILIACS IN HUNGARY

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(Received January 20, 1994)

(Accepted September 26, 1994)

Twenty-five haemophiliacs who had been infected with HIV in 1982 or 1983 were followed up from 1986 to 1993. The absolute number of the CD4+ and CD8+ cells, neopterin levels and more recently the percentage of activated, DR+ T lymphocytes were determined twice a year. In most patients a permanent decline in the CD4+ cell count was observed whereas in two HIV-infected haemophiliacs the absolute number of CD4+ cells did not change during the observation period. In these long-term non-progressor patients no clinical symptoms and no increased neopterin levels were observed. T cells subset and neopterin measurements were found to predict the development of AIDS. AIDS developed only in those patients who exhibited both a CD4+ cell count of <350/μl and a serum neopterin concentration of >20 nmol/l. A negative correlation was observed between the percentage of activated, DR+ T lymphocytes and the CD4+ cell counts.

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Until 1992 most of the about 600 Hungarian haemophiliacs were treated with cryoprecipitate and PCC preparations produced in the National Institute of Haematology and Blood Transfusion in Hungary. Both preparations originated exclusively from unpaid voluntary blood donors. In 1983–84 – because of shortage of some of the Hungarian products – about fifty patients with haemophilia were treated with imported blood products, too. According to the first HIV antibody screening of all Hungarian haemophiliacs, performed at the end of 1985, 28 patients out of those who received imported. FVIII and FIX were found to be confirmed HIV positive [1]. The remaining 22 patients who were treated with the same blood products remained HIV seronegative, none of these patients were found to seroconvert at the follow-up screening either.

The immunological follow-up investigation of the HIV seropositive haemophiliacs was started in 1986. Age-matched HIV-seronegative haemophilia patients were longitudinally tested, too. The immunological investigations were performed every six months. The percentage and absolute number of the CD3+, CD4+, and CD8+ cells were measured, and CD4/CD8 ratios were calculated. In addition, serum neopterin and beta₂ microglobulin levels, as well as the serum IgG and serum IgA levels, as predictive markers were also continuously monitored. Beta₂ microglobulin, and IgA, IgG data are not presented in the paper. In the last 2 years the relative and absolute number of activated, double labeled CD3+DR+, CD4+DR+ and CD8+DR+ cells, were also determined.

Materials and methods

Patients. The study was performed in 25 HIV seropositive haemophiliacs (8–43 years of age) and as control in 10 seronegative haemophiliacs (12–39 years of age) as well as in 23 seronegative healthy persons (23–46 years of age). Till July, 1993, full-blown AIDS developed in 10/25 seropositive patients (10) and 7 of them had died already.

Measurement of the T cell subsets. Between 1985 and 1987, the T cell subsets were counted by fluorescence microscopy, using a Leitz microscope. Since 1987 the measurements were performed by flow cytometry on FicolI-Uromiro separated peripheral blood mononuclear cells, with the commercial monoclonal antibody reagents of Ortho Diagnostics, and Becton Dickinson, or with the immunomonitoring kit of Becton Dickinson (CD3 FITC or PE, CD4 FITC or PE, CD8 FITC or PE, HLA DR PE). Becton Dickinson FACSTAR and Ortho Cytoron Absolute equipments were used.

Determination of serum neopterin level. Neopterin concentrations were quantified using a commercial radioimmunoassay [RIA Henning-Berlin FRG).

Results

Changes in the absolute number of CD4+ and CD8+ cells and the CD4/8 ratios in longitudinally (1986–1992) tested HIV seropositive, and seronegative haemophiliacs between 1986 and 1992. The HIV seropositive and seronegative haemophiliacs were tested between March 1986 and May 1993. No significant changes were found in any parameter in the seronegative group during the seven year follow-up period.

In contrast, a significant drop in the absolute number of CD4+ cells, as well as in the ratio of CD4+/CD8+ cells was observed in the HIV seropositives (Fig. 1). The rate of the decline of CD4+ cells was higher between 1986 and 1989 than during the last 4 years of observation. The absolute number of CD8+ cells showed a slight increase during the second half of the observation period.

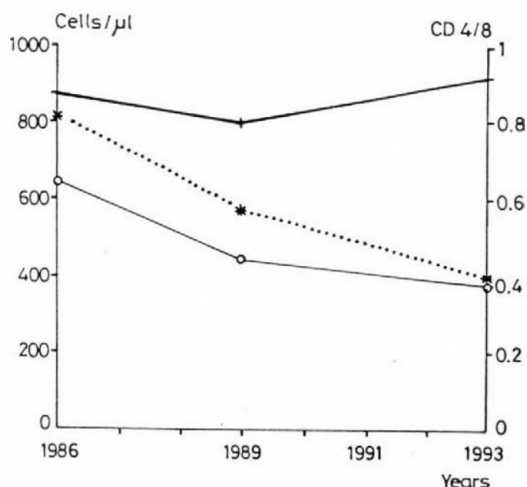


Fig. 1. Changes in the absolute number of CD4+ and CD8+ T lymphocytes as well as of CD4/8 ratio in a cohort of HIV infected haemophiliacs between 1986 and 1993. Normal values (mean±S.D.) for absolute CD4+ lymphocyte count: 924 ± 243 cells/μl, for CD8+ lymphocyte count: 508 ± 142 cells/μl, for CD4/8 ratio: 1.90 ± 0.53 ○ CD4/μl; + CD8/μl; * CD4/8

Frequency of the CD4+ and CD8+ cells in patient with normal, moderately. and strongly elevated neopterin levels. A strong negative correlation was found between the percentage of CD4+ lymphocytes and neopterin levels in HIV+ haemophiliacs. The higher the neopterin concentrations were, the lower CD4+ values were measured. In contrast, a very strong positive correlation existed between the percentage of CD8+ cells and the neopterin levels (Fig. 2).

Predictive value of the immunological investigations. In our previous studies [2], the parallel measurement of the CD4 cell numbers and neopterin levels had a high predictive value. Our present results are in agreement with this finding. In 1987, 3 HIV-seropositive haemophiliacs had CD4 cell counts less than 350/μl and a neopterin level of more than 20 nmol/l. All the 3 patients died already from AIDS. Between 1988 and 1991, CD4 values dropped below 350/μl and neopterin levels increased beyond 20 nmol/l in 4 other patients. Full-blown AIDS developed in all of these patients, two of them died already. In 14 patients, CD4 cell counts of <350/μl and neopterin levels

exceeding 20 nmol/l were never observed in parallel. None of those patients developed full-blown AIDS till mid 1993.

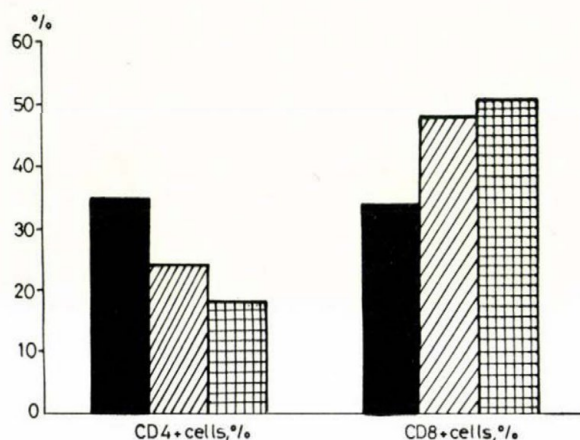


Fig. 2. Percentage of CD4+ and CD8+ cells in patients with low (<10 nmol/l), moderately elevated (10–15 nmol/l) and strongly elevated (>15 nmol/l) neopterin levels. Solid columns: neop < 10 nmol/l; shaded columns: neop 10–15 nmol/l; stippled columns: neop > 15 nmol/l

Change in the CD4+ and CD8+ cell numbers in the individual HIV+ patients.

When the trend of the changes in the number of CD4+ and CD8+ cells was analyzed in individual patients, marked differences were observed. In Figs. 3 and 4, different patterns of T cell subset changes are summarized. Figure 3A shows parallel rapid decrease in both the CD4+ and CD8+ cell counts during a relatively short, three year observation period in a boy who was 10 year old at the time of HIV infection. The extent of CD4+ drop exceeded that of CD8+ cells, that is why the CD4/CD8 ratios also decreased. In an other patient who was 5 year old at the time of HIV infection (Fig. 3B) no changes were observed in the absolute number of CD8+ cells, the CD4+ cell counts, however, dropped from 1000/ μ l to <100/ μ l in 6 years. Figure 3C shows a quite different pattern. In this patient, who was 10 years old at the time of HIV infection, the initially low CD4+ counts slowly decreased, while CD8+ cell counts increased during the 7 year's period of the follow-up study. In Fig. 4 observations in two long-term non-progressor patients are summarized. In one of these patients (22 years old at the time of HIV infection) who is still asymptomatic, not only the CD8+, but the CD4+ cell numbers exhibited a continuous elevation (Fig. 4A). In this patient every other immunological parameters (neopterin, IgG, IgA concentrations) have been in normal range during the 7 years observation period. Interestingly enough, an other patient (very close relative of the patient in Fig. 4A) showed almost the same picture

(Fig. 4B). This patient was 33 years old at the time of HIV infection and he is asymptomatic, too.

Correlation between the activated CD3+, CD4+ and CD8+ cells at the HIV+ haemophiliacs. In these experiments, peripheral blood mononuclear cells of the HIV patients were stained with a mixture of anti-CD3 (anti-CD4, anti-CD8) and anti-DR antibodies. Cells that were double labelled, that is, labelled with both antibodies, were considered as activated T cells (activated CD4+, CD8+ T cells). In each HIV-infected haemophiliacs at least 20% of CD3+ cells were found to be DR+, so-called activated cells. The median value of the CD3+DR+ activated T cells was 51.7%. The patients were divided in two groups according to the percentage of CD3+DR+ cells (lower or equal to – higher than the median value) and the absolute number of the CD4+ and CD8+ T lymphocytes as well as the percentage of the activated CD4+ and CD8+ T lymphocytes were compared (Table I). The mean absolute number of the CD4+ cells was significantly lower in the group of patients with high than in those with low percentage of activated T cells whereas no significant differences in the number of CD8+ cells were found between the two groups. The percentage of both the DR+ CD4+ and the DR+CD8+ T cells increased in parallel to the percentage of DR labelled CD3+ cells, although the extent of correlation was markedly higher in the case of the CD8+ lymphocytes (Kendall correlation coefficient for CD4+ cells: 0.448, $p=0.02$, for CD8+ cells: 0.695, $p=0.0003$).

Table I

Absolute number of the CD4+ and CD8+ lymphocytes and percentage of activated (DR+) CD4+ and CD8+ lymphocytes in HIV infected haemophiliacs with high and low** percentage of CD3+DR+ activated T lymphocytes*

	CD3+DR+ lymphocytes >51.7%	CD3+DR+ lymphocytes ≤51.7%	p***
CD4+ cells/μl	222.7±182.4	467.5±154.8	0.015
CD8+ cells/μl	1028.3±510.0	1313.0±640.0	n.s.
CD4+DR+ cells, %	36.3±22.4	20.3±5.4	0.070
CD8+DR+ cells, %	63.7±9.3	42.4±13.1	0.003

*Higher than the median value,

**Equal to or lower than the median value

***Calculated by the two-sample t-test

n.s. = not significant

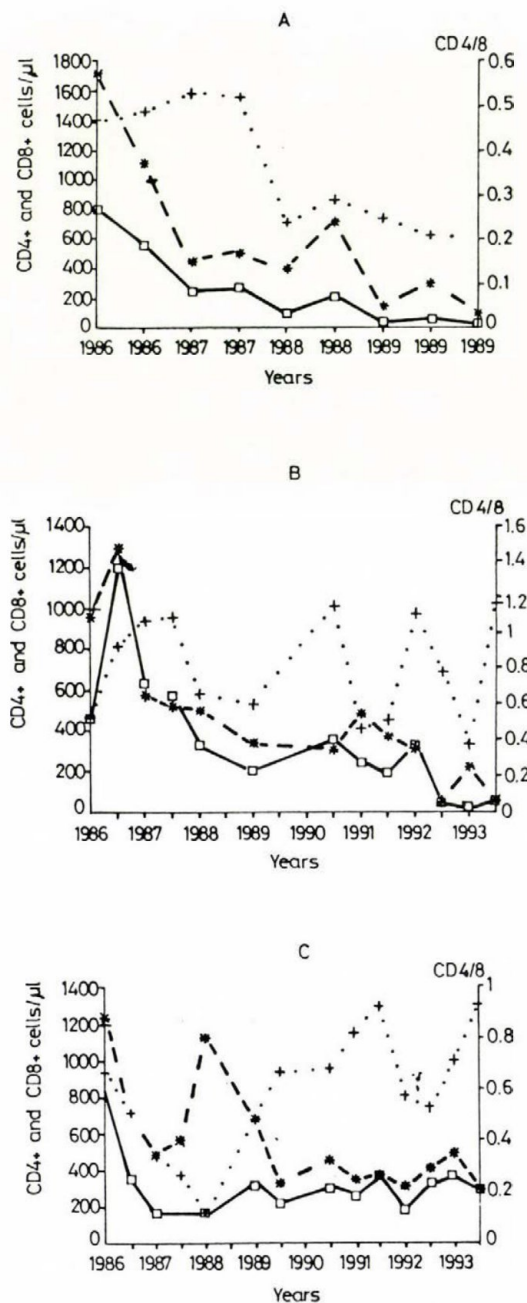


Fig. 3. Changes in the absolute number of CD4+ lymphocytes ($\square$ — $\square$), CD8+ lymphocytes (+ +) and of CD4/8 ratio (x - - - x) in three haemophiliacs (A, B, and C) with progressing HIV disease

Discussion

Previously [2] we have reported on the results of the first 42 month follow-up study of 25 HIV infected Hungarian haemophiliacs. HIV infection of these patients took place in 1982 and 1983. Our present findings, in agreement with data obtained in much larger cohorts of HIV-seropositive haemophiliacs [3–6] indicate that the drop of CD4+ cells that which were observed in the first years of HIV disease is permanent. For 1992 the mean value of absolute number of CD4+ cells in this group dropped below 400/ μ l. The rate of the CD4+ cell decline, however, was lower in the second than in the first part of observation period. This finding can be due to the death in AIDS of the relatively faster progressors from the cohort.

A similar trend in clinical progression was observed in the longitudinally tested haemophiliacs: whereas till 1989 AIDS developed in only 3 patients [2], the cumulative number of AIDS cases increased to 10 in this cohort till mid-1993. Since the date of HIV infection of each patients is well known [1], it can be calculated that the 10 year cumulative incidence of AIDS was 40% in this small group. This proportion is slightly lower than that observed in other cohorts. For example, Ragni and Kingsley [7] found that 43/84 patients with haemophilia (52.1%) progressed to AIDS at 11 years after seroconversion. Sampalis et al. observed 9/18 HIV-seropositive haemophiliacs (50%) to develop AIDS within 10 years of HIV infection [8]. The difference is most probably due to the relatively low mean age in our group: 14/25 patients were younger than 14 years of age at the time of seroconversion. Several data, including our previous studies [2, 9, 10] indicate that there is a significant negative correlation between the age of the patients and the rate of progression towards AIDS.

The present findings support our previous observations [2] on the strong predictive value of parallel measurements of CD4+ cell counts and neopterin levels in HIV infected haemophiliacs. According to our first results [2] only those patients who had CD4+ cell count less than 350/ μ l and a neopterin value of more than 20 nmol/l, in 1987 progressed to AIDS in 1989. The same types of immunological alterations were observed in 4 other patients between 1988 and 1991, all patients developed AIDS till mid-1993. By contrast, the patients whose CD4+ cell counts were more than 350/ μ l and/or their neopterin levels never exceeded 20 nmol/l did not progress to AIDS during the same period of time. These findings are in agreement with the results obtained in large cohorts of HIV-infected homosexual men [11].

The progression of HIV infection to disease is influenced by the age of patient at the time of seroconversion, and by a number of other factors that are only partly known. Accordingly, a marked heterogeneity in the time course of HIV infection had been observed in different cohorts tested. About 10% of the infected patients are asymptomatic and do not exhibit major alterations in immune functions even 10–12 years after HIV infection. These patients are called long-term survivors [12] or more recently long-term non-progressors. We have also found two patients, close relatives to each other who met the criteria of the long-term non-progressor. No decline in their CD4+ cell percentage and absolute number could be observed even 10–12 years after HIV infection. Both are asymptomatic and interestingly enough, no signs of immune activation were observed, their neopterin levels and percentage of DR+ T cells were found to be within the normal limits throughout.

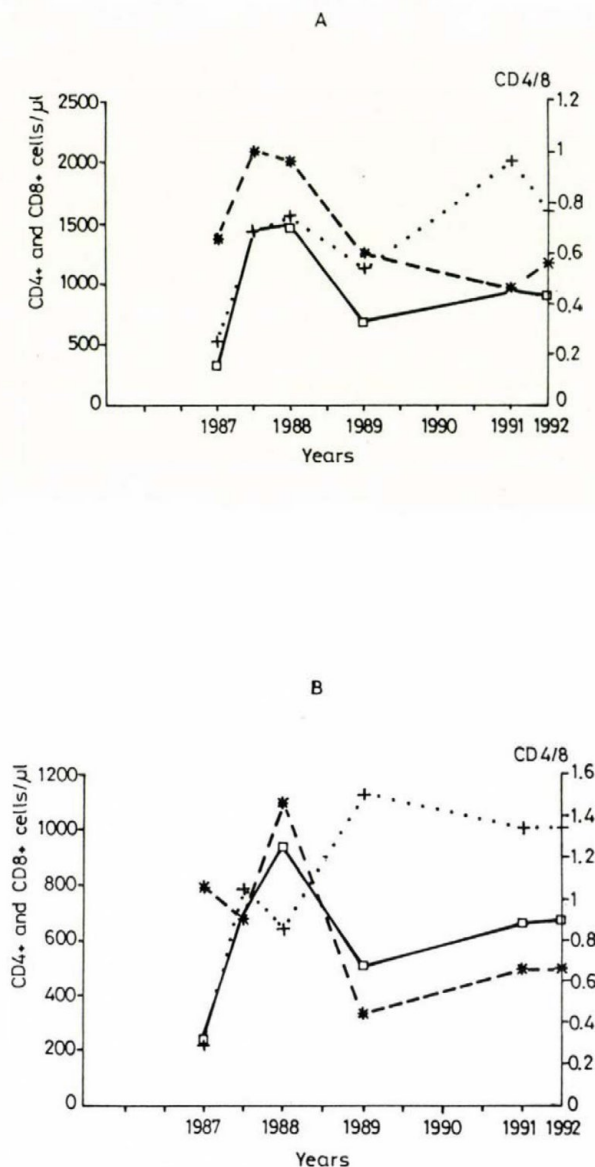


Fig. 4. Changes in the absolute number of CD4+ lymphocytes ($\square$ — $\square$), CD8+ lymphocytes (+ +) and of CD4/8 ratio (x ---- x) in two haemophiliacs (A and B) with non-progressing HIV disease

Observations obtained in these 2 patients and other members of the longitudinally tested group of HIV infected haemophiliacs support the role of immune activation in the progression of HIV disease [2, 13, 14]. Activation was not limited,

however, to the CD4+ cells, many of the CD8+ cells were found to be DR+, too [Table 1]. According to recent studies, activation of the CD8+ cells is a hallmark of HIV infection and activated CD8+ cells are detectable throughout the infection in the peripheral blood and the tissues [15, 16]. The activated phenotypes of CD8+ cells reflect diverse functional cell types. At later stages of HIV disease, some CD8+ cell functions, like CTL activity are also lost. The main reason for this could be the programmed cell death (apoptosis) which concerns both CD4+ and CD8+ cells [17], although different mechanisms may trigger apoptosis in these two types of cells [18]. Activation of T cells may directly enhance the rate of apoptosis [17]. Our future longitudinal studies to be performed in the present group of haemophiliacs and in patients belonging to other risk groups will aim to clarify the relationship between different types of activation of CD4+ and CD8+ cells on one hand, and the rate of apoptosis in cultured cells of the same patients.

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REMARKS ON THE APPEARANCE OF “VIOLINE” ON CANTAL CHEESE

(A NOTE)

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(Received March 24, 1994)

(Accepted May 30, 1994)

A mould, *Scopulariopsis brevicaulis* was isolated from a Cantal cheese. This mould was responsible for the “violine” phenomenon. Its optimum pH growth was about 7 and the mould was able to grow suitably at 10 °C, the temperature of cellar for cheese ripening. It was inhibited by high sodium chloride contents or low water activities.

The obtention of a nice rind in “Cantal” manufacturing process is one of the most important objectives of the cheese-makers. This rind has to be thick with bulges and craters penetrating to the cheese. Its colour must be golden to orange-yellow. Cheese-makers call that the “Boutonné” rind. Nevertheless, it occurs very exceptionnaly that this rind is covered transitorily with purple spots called “violine” [1], which are due to a mould. This note deals with the study of the influence of some physicochemical parameters on the growth of this mould.

Materials and methods

The strain we studied was isolated from a Cantal cheese and was identified as “*Scopulariopsis brevicaulis*” (Saccardo) Bainier.

Cultures were carried out on solid medium containing yeast extract (0.5% w/v), peptone (0.5% w/v), glucose 1% (w/v) and agar-agar (3% w/v) [2]. Except otherwise indicated they were incubated at 10 °C. This basal medium was sterilized by autoclaving at 120 °C for 20 mn.

Growth of mould was estimated by measuring the increase in colony diameters [3]. An average of 15 determinations gave Vr, the radial growth rate expressed in µm/h for each studied parameter.

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Results

Growth characteristics. At the beginning of the growth, the mould was white velvety and became pale brown after a few days. *S. brevicaulis* was able to grow on Czapek mineral medium as well with nitrate as with ammonium as nitrogen source. This mould used glucose, lactose and saccharose as carbon source but not lactic acid. It was unable to use rapeseed oil and tributyrin and did not hydrolyse gelatin.

Effect of pH. Growth rates were determined at 10 °C on the basal medium buffered to different extents: citrate-citric acid 100 mM at pH 3–4 and 5; sodium hydrogenophosphate-sodium dihydrogenophosphate 100 mM at pH 6 and 7; Tris-HCl 100 mM at pH 8 and sodium carbonate-sodium bicarbonate 100 mM at pH 9 and 10.

S. brevicaulis was able to grow suitably when the pH ranged from 6 to 10 with an optimum at pH 7 (Fig. 1A). The pH of a "Cantal vieux" cheese rind was about 7.5.

Effect of sodium chloride and water activity. Growth rates are given in function of sodium chloride content (5 to 20% w/v) (Fig. 1B). It was found that at 5% NaCl, corresponding to water activity of 0.972 [4], the growth rate of *S. brevicaulis* was optimal (137% of growth without NaCl). This growth activation phenomenon was previously observed for other moulds [5]. Then growth rates decreased linearly when sodium chloride content increased. Growth was null at 20%. With sorbitol, this growth activation phenomenon was not observed for A_w equal to 0.972. Nevertheless up to A_w equal 0.914 the both effects (NaCl and sorbitol) were quite identical. Sodium chloride content of Cantal cheese was about 2.5% (w/w).

Effect of temperature. Results are given in Fig. 1C. The optimum temperature for growth was found to be about 18 °C. At 10 °C, which is the temperature of cellar where ripening is carried out, growth was about 80% of the maximal rate. At 4 °C, growth was null.

Inoculation of industrial cheese. Cheeses were inoculated with 10^6 spores of *S. brevicaulis* per litre of used milk at two distinct steps of cheese manufacturing: inoculation of "Caillé frisé" and inoculation of the "toile". The mould rapidly invaded the surface of the cheese and gave a purple and crackled rind.

Conclusion

The main characteristics of cheese (pH, water activity and ripening temperatures) do not make possible the automatic elimination of *S. brevicaulis*. Nevertheless, it has to be noticed that this mould was unable to grow when the natural microflora was abundant and well developed. The appearance of this mould was more often than not weak and transitory. In fact, when raw milk is used, a correct growth of usual flora is generally sufficient to provide a good protection. Of course, important and abnormal inoculations have to be avoided. Traditional cheese manufacturing warrant a good growth of normal flora.

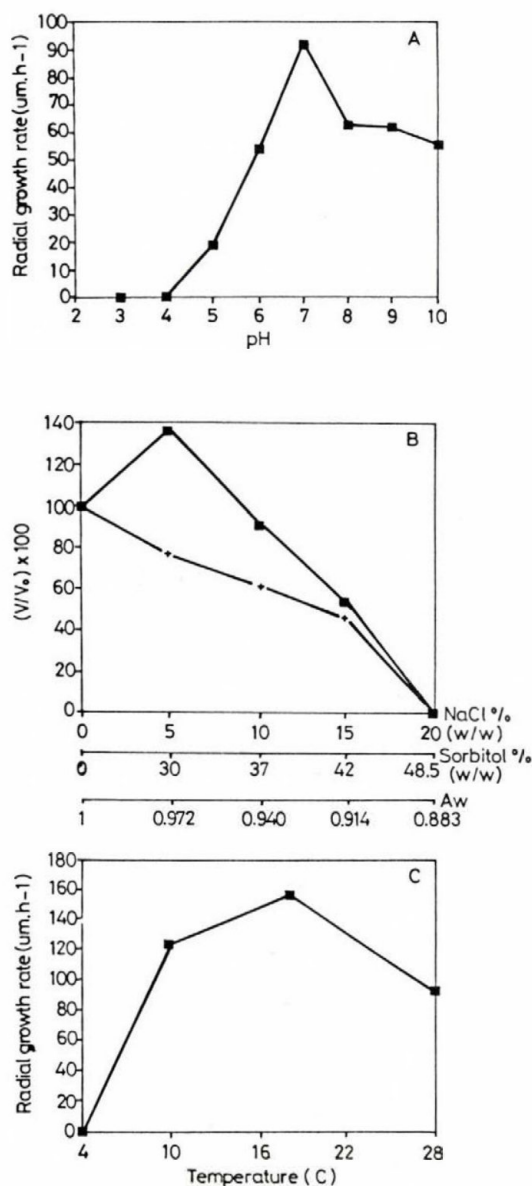


Fig. 1. Growth parameters of *S. brevicaulis*. A: influence of pH; B: influence of water activity on growth; V: growth rate with NaCl (■) or sorbitol (+); V_o: growth rate without NaCl nor sorbitol; C: influence of temperature

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IMMUNOMODULATING EFFECT OF ACRIDINE TAUTOMERS ON EUKARYOTIC CELLS

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(Received June 3, 1994)

(Accepted August 9, 1994)

The immunomodulating effect of some new amino- and imino-acridine derivatives, were investigated on antibody dependent cellular cytotoxicity (ADCC) and induced-blast transformation of lymphocytes. In different concentrations (2.0×10^{-6} M, 4.0×10^{-8} M and 2.0×10^{-5} M) the drugs produced a suppression of PHA and ConA induced cell proliferative response except in the case of 2b, 2d and 2g amino-acridines. The suppressive effects were dose dependent and exhibited a higher inhibitory level in the case of imino-acridines. Some drugs at low concentration exerted a little enhancing effect on ADCC reaction.

It is known that the most specific agents against *Trypanosoma* and *Plasmodium* spp. are antibodies produced by the immune system. However, this is the less effective mechanisms in vivo, because immunosuppression occurs in both types of diseases. Considering these immunosuppressive effects of parasitic infections and the present therapeutic possibilities, the newly synthesized acridine derivatives were screened for immunomodulating or stimulating effects as a possible tool for chemotherapy.

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Materials and methods

Chemistry. The 9-aminoalkyl and 9-imino-10-alkylacridines listed in Table I were prepared by alkylating 9-aminoacridine, under phase transfer catalysis conditions [1]. The synthetic pathway is schematized in Fig. 1. Separation of isomers was achieved by column chromatography on silica gel using methanol:ammonia (90:10 v/v) as eluent.

Biology. The compounds were dissolved in DMSO; final concentrations were 2.0, 4.0 and 20.0×10^{-6} M, in all triplicate test cultures. Both control cultures and test cultures contained DMSO in the same concentration.

Isolation of mononuclear cells. Human peripheral blood mononuclear (PBM) cells were isolated from healthy blood donors by Ficoll-Uromiro gradient centrifugation. The cells were washed and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine. To provide monocyte-free (Mo-PBM) cell suspension, the freshly drawn heparinized blood was treated with carbonyl iron powder at 37 °C for 5 min and then lymphocytes were isolated on Ficoll-Uromiro gradient.

Lymphocyte blast transformation (T lymphocyte proliferation assays). Mononuclear cells were routinely cultured in flat-bottom Greiner microtiter plates at 2×10^5 cells (0.2 ml/well). The triplicate cultures containing the drugs were incubated in the presence or absence of mitogens: PHA 1:200 diluted stock solution; ConA 5 µg/ml in a CO₂ incubator for 72 h. For the determination of lymphocyte DNA synthesis, 0.5 µCi 3-H-thymidine was added to all cultures for the last 5 h and the cells were collected on GFC filter paper. The amount of radioactivity incorporated was determined in a liquid scintillation counter. The results are expressed as difference of cpm between the incorporated activity of transformed cells and control cells (without mitogens) [2].

Antibody dependent cellular cytotoxicity (ADCC) test [3]. Human O Rh-positive red blood cells were used as target and PBM or Mo-PBM cells as effector in 1:10 ratio. The reaction was mediated by red blood cell specific anti-D antibody. The cultures were incubated at 37 °C for 16 h and the amount of released ⁵¹Cr in each supernatant was determined with gamma-counter. From the average of triplicate released values, the percentage of cytotoxicity was calculated according to the following formula.

$$\% \text{cytotoxicity} = \frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr releases} - \text{spontaneous } ^{51}\text{Cr release}}$$

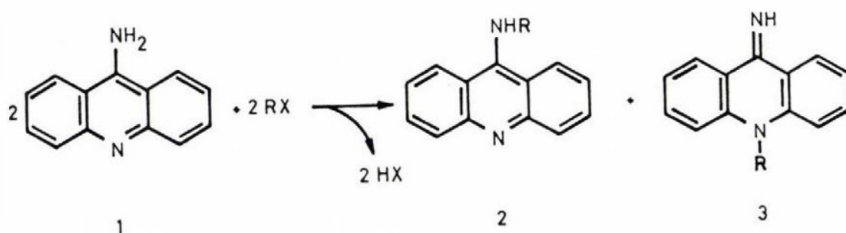


Fig. 1. Synthetic pathway schematizing alkylation of 9-amino acridine under PTC conditions

Table I

Chemical data of acridine derivatives

Compounds	mol wt
1 9-aminoacridine	230.5
2a 9-ethylaminoacridine	258.5
3a 9-imino-10-ethylacridine	258.5
2b 9-propylaminoacridine	272.5
3b 9-imino-10-propylacridine	272.5
2c 9-butylaminoacridine	286.5
3c 9-imino-10-butylacridine	286.5
2d 9-isopropylaminoacridine	272.5
2e 9-pentylaminoacridine	264
3e 9-imino-10-pentylacridine	300.5
2f 9-(2,-dimethylaminoethylamino)acridine	301.5
3f 9-imino-10-(2,-dimethylaminoethyl)acridine	301.5
2g 9-(3,-diethylaminopropylamino)acridine	343.5
3g 9-imino-10-(3,-diethylaminopropyl)acridine	343.5

Results

Data obtained for four different blood donors represent a single experiment. Donor-to-donor variation was not significant. In the case of blast transformation induced by PHA and conA the results were expressed as a percent of the control cultures which contained the same DMSO as test cultures. The cell viability with the drugs after 4 h incubation was more than 98% (trypan blue indication) (Table II).

The majority of the drugs showed a dose dependent inhibition, but at low concentration (2.0 or 4.0×10^{-6} M) the degree of the suppressive effect was slight and with drugs 2b, 2d and 2g there was slight enhancing effect on the blast transformation stimulated by mitogens. The suppressive effect at 2.0×10^{-5} M concentration was significant in every cases.

The ADDC activity of K cells was tested at 1:10 target/effector ratio (Table III). Some enhancing effect occurred at low concentrations with drugs 1, 2a, 2c, 3c, 2d, 2e, and 2f. Over these concentrations (at 2.0×10^{-5} M) all reactions were inhibited. The monocyte free PBM (Mo-PBM) was as effective as the whole blood, that is, the lymphocytes were responsible for the effects.

Table II

*Effect of acridines on lymphoblast transformation stimulated by PHA and ConA**

Drugs	Mitogens, concentration $M \times 10^{-6}$					
	PHA			ConA		
	2.0	4.0	20	2.0	4.0	20
1	63	34	8	63	60	11
2a	57	56	28	80	58	17
3a	56	32	6	59	26	2
2b	129	124	36	136	104	71
3b	52	45	10	85	90	20
2c	66	53	2	121	68	24
3c	69	39	9	95	60	19
2d	131	82	34	198	116	33
2e	52	55	16	107	113	29
3e	34	44	7	93	94	39
2f	81	74	21	185	123	51
3f	45	34	8	65	60	13
2g	150	122	73	139	73	42
3g	110	76	19	109	70	27
Control values: 22180 ± 4100 cpm/ 10^5 cell				16660 ± 4200 cpm/ 10^5 cell		
without DMSO: 24610 ± 3460				18415 ± 3110		

*Results are expressed as percent of the control values

Discussion

There are benz(c)-acridine derivatives with mutagenic and carcinogenic effects [4]. However, some substituted acridines act as anticancer agents [5] while others have chemotherapeutic effects on protozoa. [6] at the same time. It is well known that some of the protozoal infections reduce the immune response of the host. Therefore we have been searching for antiprotozoal compounds that are not immunosuppressing; even some of them have been found to enhance the immunoreponse of human leukocytes [7]. Apparently this immunostimulating effect depends on the chemical structure of substituted acridines. The enhancing effect could be due to lymphocyte interactions mostly in the case of amino-acridines. The present results also indicated that imino-acridines (3a, 3b, 3e, 3f and 3g) had remarkable immunosuppressive effect compared to that of amino acridines. The PHA induced blast transformation of lymphocytes is more important because both cellular and humoral immune response of T and B cells are involved in the host defense mechanisms and antibody production.

Table III

Effect of acridines on ADCC reaction (%)

Drugs in $M \times 10^{-6}$	Mo-PBM		PBM	
	test value: 29		test value: 18	
	2.0	4.0	2.0	4.0
1	22	17	19*	13*
2a	26*	21*	16	12
3a	19	16	15	11
2b	20	15	15	14
3b	19	14	13	10
2c	27*	31*	16	19*
3c	31*	23*	20*	13
2d	24*	19	20*	17*
2 e	23	21*	20*	13
3e	20	18	15	10
2f	26*	18	20*	12
3f	22	18	15	13
2g	19	14	15	12
3g	18	13	14	10
Control value:	23	20	16	12

Target/effector ratio, 1:10

The suppressive effect of the acridines at the 2.0×10^{-5} M concentration was higher than 70% in every case

*Enhancing effect of the drugs

The data reported here with ADCC reaction also confirm the cytotoxic reaction both of the PBM and monocytes free PBM. Both cell subsets are effectors of ADCC against sensitized human erythrocyte target cells and the effects of the drugs indicate some enhancing effect on both cell types of the amino-acridines 2a, 2c, 2e, 2f and 2d. Only one of the imino-acridine (3c) had the same enhancing effect – other compounds were immunosuppressive.

Acknowledgements. This work was supported by the Hungarian Research Fund (OTKA 2703) grant and grant from the European Cost Acrival 815 Programme ERB CIPECT, No. 92 60 43.

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THE EFFECT OF VASOPRESSIN ON THE PHOSPHOINOSITIDES OF *TETRAHYMENA* *PYRIFORMIS*. RELATIONSHIP WITH GLYCOGEN CONTENT

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(Received June 13, 1994)

(Accepted June 27, 1994)

In *Tetrahymena pyriformis* a 20% decrease of ^{32}P -PIP₂ was produced by 10^{-6} M vasopressin treatment within 30 s. A 17% decrease occurred in the content of PAS positive material within 5 min after administration. The effect of vasopressin was similar to that of hepatocytes.

It is well known that different hormones characteristic of higher organisms can be found even in lower animals especially in *Protozoa* [1]. Moreover, some of these hormones have specific effect on *Tetrahymena pyriformis*. Hormonal imprinting means that pretreatment with a hormone alters the binding capacity and response of the cell measured after a second treatment by the same hormone [2].

Several works confirmed the presence and function of different elements of signal transduction like cAMP-adenylate cyclase, Ca-calmodulin system [3]. Recently many data have been described for the existence and function of phosphatidylinositol system in protozoa [3, 4]. It seems to take part in signal transduction similarly to that in higher organisms.

The recent work attempts to confirm and extend previous observations. The effect of vasopressin was investigated on *T. pyriformis*.

Materials and methods

Culturing and treatment. *T. pyriformis* GL cells were cultured in 1% Bacto Tryptone medium containing 0.1% yeast extract (Difco, Michigan, USA) at 28 °C for 48 h. One bottle of culture (150 ml) serving as control was not pretreated, the other was pretreated with 10^{-6} M vasopressin (Vasopressin-Sandoz, Sandoz Pharma LTD, Basle, Switzerland) for 1 h. Both cultures were centrifuged, washed and returned to plain medium for 24 h. Before the second treatment the cells were prelabelled with ^{32}P -Na orthophosphate (Izinta, Budapest) for 120 min at 28 °C. The cells were thoroughly washed and both

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cultures were separated into two groups. Five ml samples were taken from each culture. Samples of one of the series were not treated, samples of the other one were treated with 10^{-6} M vasopressin for different periods (0, 30, 60 s, 5 min). In all cases two replicas were performed.

Lipid extraction. The treatment was stopped by 10% ice-cold TCA and the samples were kept at 4 °C for 30 min. After centrifugation the pellet was extracted with 2 ml chloroform:methanol:HCl (100:200:1 v/v) at room temperature for 15 min. Then 0.66 ml chloroform and 0.66 ml water were added to partitionate the extract. At last the samples were centrifuged for better separation. The lower lipid phase was dried in N_2 flow.

Lipid separation. The samples were resuspended in chloroform:methanol (6:1 v/v), and applied to Silica-Gel plates. The phospholipids were separated by one-dimensional two-step thin-layer chromatography [5]. The chromatogram was covered with X-ray film which was developed after 36 h exposure to detect phospholipid spots. The thin-layer chromatogram was also developed in I_2 vapor to detect the standards (Calbiochem, Luzern, Switzerland). Then the identified spots were scraped into vials and scintillation cocktail was added to them. The radioactivity was measured in a Beckman LS 9000 scintillation counter.

Measurement of PAS positive (glycogen) content. Culturing and pretreatment (1 h, 10^{-6} M) of the cells was the same as above. The cultures were separated into control and vasopressin treated groups. Samples were taken at 0, 1, 5, 15, 50, 60 min after vasopressin addition. The cells were fixed in PBS-formalin (4%) washed out, dropped on slides and periodic-acid Schiff (PAS) reaction was performed. The staining intensity of the cells was measured by a Zeiss Amplival cytophotometer at 554 nm and recorded by a Zeiss K linear compensation recorder. In all groups 20 cells were tested. The experiment was performed six times.

Results and discussion

In vitro and in vivo experiments demonstrate that in higher organisms several different cells have vasopressin receptors, coupled to different transmembrane signal system [6–10]. V_1 vascular vasopressin receptor is coupled to G_q protein, phospholipase A2, C and D, Ca channels [6, 11]. Beside its antidiuretic effect, vasopressin induces glycogen breakdown in rat liver cells [12, 13]. In humans vasopressin has only a minor role in the regulation of hepatic function compared to rats [14]. Vasopressin is able to regulate hepatic regeneration in rats [15].

The effects of vasopressin to hepatocytes is mediated by phosphoinositides. In different experiments made on isolated hepatocytes vasopressin caused about 15–30% decrease in phosphatidylinositol 4,5-bisphosphate (PIP_2), 10–20% decrease in phosphatidylinositol 4-phosphate (PIP) and a moderate, 5–10% or no decline in phosphatidylinositol (PI) content. The maximum effect was obtained at 15 or 30 s of vasopressin stimulation. The hormone was effective in 10^{-6} – 10^{-8} M [5, 9]. In renal medulla vasopressin stimulated phosphoinositide hydrolysis is blocked by V_1 receptor antagonists but not by V_2 antagonists [7, 10]. It suggests that V_1 type receptor are linked to PI system.

Protozoa contain hormones that are characteristic of higher organisms, moreover they are able to give specific response to them [2]. It was shown that 10^{-6} M vasopressin changed the activity of the contractile vacuole in *Tetrahymena*, i.e. it had effect on water regulation, like in higher organisms [16].

The number of data on existence and function of phosphoinositol system in protozoa is increasing. In a review the effect of temperature, alcohols, drugs and

exogenous phospholipid head group has been discussed [4]. It was shown that insulin changed the phospholipid composition of two different taxa of *Tetrahymena* [17]. An other study provides some insight on insulin effect as increased the hydrolysis of PIP₂ [3].

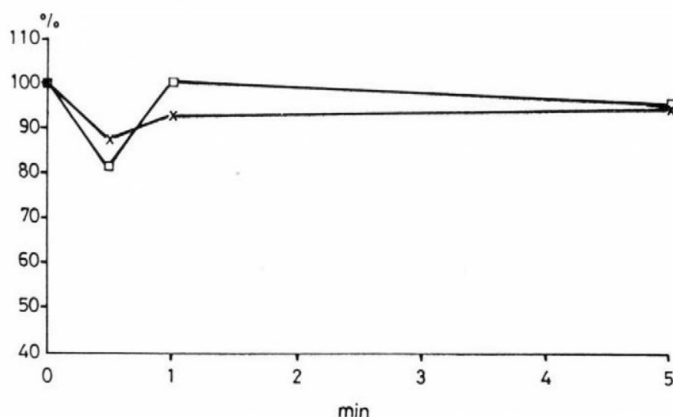


Fig. 1. Time course change of ³²P labelled PIP₂ after vasopressin treatment. The values are as per cent of control (□ = unstimulated cells incubated for the same length of time) radioactivity. The results are the mean of four separated experiments. In the vasopressin pretreated group (×) the cells were treated twice (as detailed in Materials and methods). The decrease at 30 s is significant

In our experiment in *Tetrahymena* 10⁻⁶ M vasopressin caused a 20% loss of PIP₂ within 30 s of treatment (Fig. 1). At 60 s and 5 min of hormone administration the level of PIP₂ approached the value of control. This time course of PIP₂ is very similar to the response given by vasopressin induced hepatocytes [9]. The quantity of PIP has not changed significantly.

The phenomenon of hormonal imprinting is known not only in higher organisms but at lower phylogenetic level, in protozoa. Hormonal imprinting means that the binding of, and a response to, a hormone is higher if the cell has previously met the same hormone [2]. In our case the PIP₂ breakdown was not higher due to the pretreatment (Fig. 1).

T. pyriformis contains a high amount of glycogen. A study has demonstrated that insulin and its A and B chain increased the quantity of PAS-positive material (glycogen) in these cells [18]. Results of several investigations indicate that vasopressin is a glycogenolytic hormone stimulating glycogen phosphorylase activity of hepatocytes and increasing the glucose release [12–14, 20].

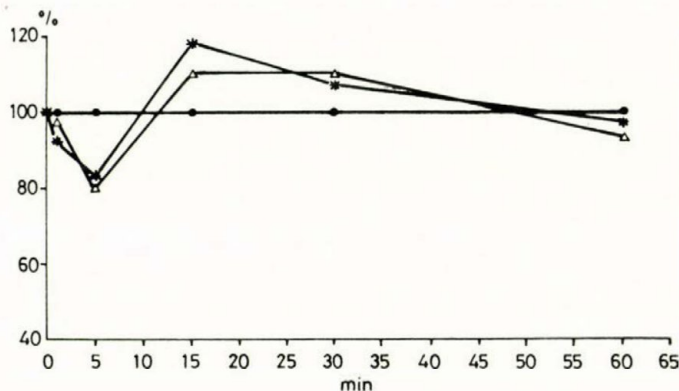


Fig. 2. Time course change in PAS positive material due to 10^{-6} M vasopressin treatment. C/C = control group ($\bullet$), C/V = once vasopressin treated group ($*$), V/V = twice vasopressin treated group (Δ). Results are expressed in per cent of 0 min and the per cent of control (same time of treatment). The values are the mean of 4 or 6 experiments. In each of them 20 cells were measured. Both values of 5 min are significant. At 15 min the C/V value is significant

We measured the change of the quantity of PAS-positive material after different time of vasopressin administration (Fig. 2). Our data show that the PAS positive substance content was 17% lower in vasopressin treated (C/V) than in control cells (C/C) after 5 min of vasopressin treatment. At 15 min the cells contained significantly more PAS positive material due to the treatment.

In the pretreated group (V/V) we measured similar PAS positive material content at 5 min as in the once vasopressin treated group, but at 15 min the increase was not significant.

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A MODEL FOR TESTING DRUG SUSCEPTIBILITY OF *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS* GROWN IN BIOFILMS ON MEDICAL DEVICES

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(Received July 18, 1994)

(Accepted August 9, 1994)

UV-sterilized polyethylene rings infected with *Pseudomonas aeruginosa* or with *Staphylococcus aureus* were implanted into artificial wounds of mice. During 5 days incubation biofilm was formed on the plastic surfaces. To determine the Minimal Bactericidal Dose on sessile bacteria, rings with biofilm were removed and incubated in Luria broth containing serial dilutions of different antibiotics. Parallel, the sensitivities of planktonic phase organisms were also determined using cells grown in broth. The biofilm mode of growth strongly reduced the sensitivity of the strains against most of the antibiotics used, especially against polymyxin B. On the other hand, β -lactam type antibiotics were equally effective against bacteria both in the sessile and planktonic phase of growth.

It is known that microbes in a biofilm mode of growth are protected by the biofilm matrix against antibodies, phagocytosis, as well as against biocides and antibacterial drugs [1]. Materials such as rubber, plastic, or metals – often used as medical implants – can provide a suitable surface for the biofilm formation, resulting an infectious focus maintaining a chronic or relapsing infection.

Previously we showed [2, 3] that *Escherichia coli* forming biofilm in the intestinal tract of mice as part of the normal flora, or as monocontaminant were less susceptible to various antibiotics than cultures grown in broth as planktonic phase cells. The only notable exceptions were β -lactam antibiotics penetrating the biofilm very effectively.

This approach, however, is not relevant in investigating bacteria often producing biofilms on medical implants. Here we report on the development of a model system for studying the influence of biofilm formation on plastic surfaces by *Pseudomonas aeruginosa* and *Staphylococcus aureus* on the antibiotic sensitivity of these bacteria.

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Materials and methods

Strains. *Pseudomonas aeruginosa* strain HNCMB 170006 and *Staphylococcus aureus* HNCMB 112002 were obtained from the Hungarian National Collection of Medical Bacteria, Budapest, Hungary.

Media. Strains were cultivated in Luria broth (LB) or on LB agar plates.

Mice. Outbred SPF mice (LATI, Gödöllő, Hungary) were used.

Antibiotics. For *P. aeruginosa* polymyxin B (Pfizer, France), gentamicin (Chinoin, Hungary), and cefoperazone (Biogal, Hungary) were used, while in experiments with *S. aureus* gentamicin, erythromycin (Lilly, USA), oxytetracycline (Chinoin, Hungary), and carbenicillin (POLFA, Poland) were chosen.

Plastic implants. Two mm thick rings were cut of a polyethylene tube 5 mm in diameter providing a surface of cca. 140 mm². The rings were sterilized by UV-light and were artificially contaminated by placing them into LB inoculated with *P. aeruginosa* or *S. aureus*. Cultures were grown overnight at 37 °C, then rings were removed, gently rinsed in Saline and were implanted subcutaneously via a small incision made on the back of mice. The wounds were closed by ligature and the animals were given feed and water *ad libitum*. Parallel, devices treated in the same way were used to determine the level of initial contamination of the rings. Adherent bacteria were removed by vigorous shaking for one minute on a Vortex apparatus (CSAV T 5001/II, 30 W) in five ml LB and the number of colony forming units (c.f.u.) per ml were determined. This contamination protocol resulted in cca. 10⁴ adherent bacteria per mm² rings to be implanted.

Persistence of subcutaneous infections. Mice were inoculated either by inserting a contaminated plastic ring subcutaneously as described above, or by injecting broth cultures containing cca. 10⁸ germs into the wound. Incisions were closed by ligature. Every five days a group of five animals were sacrificed, the rings removed and processed as above to determine the number of c.f.u. values associated with the implants. In case of animals infected with broth cultures opened wounds were rinsed with 0.5 ml broth, and the number of surviving germs in the subcutaneous tissues were determined from these lavages.

Minimal bactericidal dose. After 5 days of incubation mice were killed and the polyethylene rings were removed under sterile conditions. To measure the sensitivity of sessile phase bacteria, the rings were gently rinsed in saline and were placed into LB with or without serial dilutions of the antibiotics to be tested. After three hours incubation at 37 °C the rings were transferred into tubes containing 5 ml of LB without antibiotics. The tubes were subjected to a maximal energy vortexing. Plating serial dilutions onto LB agar plates the c.f.u. values were determined. The Minimal Bactericidal Dose (MBCD) was defined as a lowest antibiotic concentration from which equal to or less than 100 germs/ml could be recovered. To determine the effect of each given antibiotic concentration 5 rings were used.

Sensitivity of planktonic cells was investigated by adding 0.05 ml broth cultures to 2 ml of serial antibiotic dilutions in LB. Tubes were incubated at 37 °C and the MBCD values were determined as above.

Statistical analysis. Student's "t" tests was used.

Results

Persistence of the bacterial infections. Five days after infection, no *P. aeruginosa* cells (<10² germs) could be recovered from the site of inoculation of mice injected subcutaneously. The *S. aureus* strain exhibited somewhat more extended subcutaneous survival, the site of inoculation being cleared of bacteria within 10 days

after the injection. On the other hand, when either *P. aeruginosa*, or *S. aureus* were growing as biofilms on the implanted plastic surface – after a short initial increase – the germ counts became relative stable upto the 10–15th post-infection day. These results indicated that the 5 days incubation period was sufficient to colonise the plastic device and to establish a micro-environment protective against the clearing mechanism of the host, which is highly characteristic of biofilms. Therefore the model seemed to be suitable to investigate how effective the different antibiotics are against bacteria growing under similar conditions.

Sensitivity to antibiotics. While the MBCD value of polymyxin B against *P. aeruginosa* in planktonic phase was 3.12 µg/ml, the same strain exhibited a very high level of resistance against this antibiotics when growing in biofilms (MBCD=400 µg/ml) (Table I). The biofilm mode of growth increased the level of resistance by 16 against gentamicin, as well, with MBCD values of 80 µg/ml *versus* 5 µg/ml, respectively (Table I). Contrary to this, no significant difference was observed between the cefoperazone MBCD levels against bacteria growing under different conditions.

Table I

Drug sensitivity of P. aeruginosa in biofilms produced on polyethylene rings implanted into mice and in the planktonic phase

Experimental data	Antibiotics		
	Polymyxin B	Gentamicin ($\bar{x} \pm \text{SD}$ germs per mm ²)	Cefoperazone
Initial germ count	$2.0 \pm 1.0 \times 10^3$	$3.1 \pm 1.8 \times 10^4$	$2.4 \pm 1.5 \times 10^4$
Terminal germ count	$1.3 \pm 1.1 \times 10^5$	$7.6 \pm 1.1 \times 10^4$	$3.9 \pm 2.7 \times 10^5$
Student's "t" P values	0.008	0.072	0.083
MBCD			
planktonic	3.12 ± 0.78 µg	5.0 ± 2.5 µg	3.12 ± 0.78 µg
sessile	400.0 ± 100 µg	80.0 ± 20 µg	1.56 ± 0.78 µg
Student's "t" P values	0.002	0.002	0.070
Planktonic: sessile rate	1:128	1:16	~ 1:1

Sterilized polyethylene rings (surface, 140 mm²) maintained in growing culture were implanted subcutaneously into mice (initial germ count). After 5 days the mice were killed, rings removed (terminal germ count) and placed into dilutions of the antibiotic at 37 °C for 3 h. After treating with vortex, diluting, plating, and the MBCD (minimum concentration of the drug killing all bacteria over 10² germs was determined. In parallel experiments the MBCD for broth cultures was determined

When *S. aureus* was investigated in the same experimental setup, the biofilm mode of growth caused a clear cut, significant reduction of drug sensitivity against gentamicin, erythromycin, and oxytetracycline (sessile:planktonic MBCD ratios were

13, 7, and 8, respectively) (Table II). The β -lactam antibiotic carbenicillin, however, was equally effective against cells grown under either conditions (Table II).

Table II

Drug sensitivity of S. aureus in biofilms produced on polyethylene rings implanted into mice and in its planktonic phase

Experimental data	Antibiotics			
	Gentamicin	Erythromycin ($\bar{x} \pm \text{SD}$ germs per mm^2)	Tetracycline	Carbenicillin
Initial germ count	$3.7 \pm 2.6 \times 10^3$	$6.6 \pm 5.0 \times 10^4$	$1.4 \pm 0.7 \times 10^4$	$3.8 \pm 2.4 \times 10^3$
Terminal germ count	$3.3 \pm 1.2 \times 10^5$	$1.3 \pm 0.8 \times 10^5$	$2.5 \pm 0.6 \times 10^5$	$3.9 \pm 2.1 \times 10^5$
Student's "t" P values	0.008	0.081	0.003	0.033
MBCD				
planktonic	$6.25 \pm 1.56 \mu\text{g}$	$12.5 \pm 3.12 \mu\text{g}$	$3.12 \pm 0.78 \mu\text{g}$	$6.25 \pm 1.56 \mu\text{g}$
sessile	$80.0 \pm 20.0 \mu\text{g}$	$160.0 \pm 40.0 \mu\text{g}$	$50.0 \pm 12.5 \mu\text{g}$	$6.25 \pm 1.56 \mu\text{g}$
Student's "t" P values	0.003	0.003	0.002	~1
Planktonic: sessile rate	1:13	1:7	1:8	1:1

For explanation see footnote to Table I

Discussion

The formation of biofilms on implanted medical devices is a frequent phenomenon. In an otherwise hostile environment the biofilm mode of growth might provide the microbe with a favourable niche protecting against the effector mechanisms of the immune system, as well as antimicrobial drugs. According to Costerton et al. [4] the extracellular matrix of biofilms produced by different microbes represents 73–98% of the biofilm mass. The numerous charged binding sites in the matrix polymers could give a significant protection against antibiotics. Another possibility rendering bacteria more resistant could be the result of phenotypic changes in the cell envelope proteins or alteration of certain enzymatic activities when living in the biofilm micro-environment [5, 6].

Biofilms formed on artificial devices could serve as foci [7] of infection releasing planktonic cells into the tissues or into the circulation. While drug therapy could be effective against these planktonic cells, often it does not eliminate the focus itself. Due to technical difficulties, new antibiotics are tested and selected against planktonic phase cells only, and limited data are available only on the capability of various drugs to reach senile cells living in biofilms.

Using a mouse intestinal model system, we have shown that there are significant differences between antibiotics concerning their capabilities to penetrate the biofilm matrix, and to be effective against bacteria living inside [2, 3]. It was demonstrated that β -lactam antibiotics are much more effective in penetrating the polymer mass of the biofilm colony than other groups of antibiotics. Similar conclusions were reached by Vergères and Blaser [8].

Our present data show that the implanted polyethylene ring model is a suitable tool to study the biofilm formation on medical devices. The bacterial population persisting on the contaminated plastic surfaces exhibited several characteristics of bacteria living in biofilm. They could resist the clearing mechanisms of the host defence system and also became protected to a large extent against a number of antibacterial drugs commonly used in the medical practice. Similarly to our previous experience [2, 3] also in this model β -lactame drugs were the most effective. We consider the plastic implant model as an easy and more relevant alternative for mouse intestinal model in testing the capability of various drugs against biofilm-grown microbes.

Acknowledgements. This work was supported by the Scientific Research Council, Hungarian Ministry of Welfare (T559/1990) and by the Hungarian Research Fund (OTKA) grant 49.

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BIOFILMS PRODUCED BY *PSEUDOMONAS AERUGINOSA* AND BY *STAPHYLOCOCCUS AUREUS* ON MODEL MEDICAL DEVICES

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(Received July 18, 1994)

(Accepted August 9, 1994)

Polyethylene, teflon, tygon, polypropylene, silicon rubber, and rubber tubes or rings contaminated with *Pseudomonas aeruginosa* or *Staphylococcus aureus* were implanted subcutaneously into mice. After 5 days the colony forming units developing on, and attaching to them were determined. The highest numbers of bacteria were observed on rubber and silicon rubber, polyethylene was next in order, while significantly lower values were obtained on teflon and on tygon and polypropylene. Rubber devices were better colonized after heat than after UV sterilization. The number of bacteria rose further, if the already used rubber implant was resterilized, recontaminated and reimplanted. The model seems suitable to test the development of bacterial biofilms on different materials pretreated in different manners.

The production of biofilm on medical devices made from rubber, plastic or even metals has considerable medical importance [1]. Previously we developed a model [2] to study the above phenomenon using small polyethylene rings contaminated with *Pseudomonas aeruginosa* or with *Staphylococcus aureus* and implanted subcutaneously into mice. The development of biofilms were confirmed by the persistence of bacterial infections and by the enhanced resistance to some antibiotics comparing to the that of *in vitro* growing planktonic phase bacteria. In this paper we present data for biofilm formation on some plastic materials as compared to rubber, i.e. the classic material of medical devices.

Materials and methods

Strains. *Pseudomonas aeruginosa* strain HNCMB 170006 and *Staphylococcus aureus* HNCMB 112002 were obtained from the Hungarian National Collection of Medical Bacteria, Budapest, Hungary

Media. Strains were cultivated in Luria broth (LB) and on LB agar plates.

Mice. Outbred SPF mice (LATI, Gödöllő, Hungary) were used.

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Rubber and plastic implants. Rubber and plastic tubes were cut to give surface areas of 123 mm² (rubber), 140 mm² (polyethylene), 50 mm² (teflon), 72 mm² (softened polyvinyl chloride=tygon), 37 mm² (polypropylene), 18 mm² (silicon rubber). The tubes were sterilized by UV-irradiation and were contaminated by placing them into LB inoculated with *P. aeruginosa* or *S. aureus*. Cultures were grown overnight at 37 °C, then the tubes were removed, gently rinsed in saline and implanted subcutaneously via a small incision made on the back of mice. The wound was closed by ligature and the animals were given feed and water *ad libitum*. Parallel, contaminated were used to determine the initial colony forming unit values. Implanted model devices were removed after 5 days from the wounds of sacrificed mice, rinsed gently in saline and the adherent bacteria were removed by vigorous shaking for one minute on a Vortex apparatus (CSAV T5001/II, 30 W) in five ml LB. After diluting and plating the c.f.u. values per mm² were determined.

In case of rubber devices the efficiency of biofilm development was compared after UV and heat (100 °C, 30 min.) pretreatments. The effect of reusing the rubber device was also tested: in this case removed tubes from mice were sterilized by heat, recontaminated and reimplanted for a further 5 days.

Statistical analysis. Student's "t" test was applied

Table I

Adherence of Pseudomonas aeruginosa on rubber and plastic model devices implanted into mice

	c.f.u. values per mm ²					
	Rubber	Polyethylene	Teflon	Tygon*	Polypropylene	Silicon rubber
	1.5×10 ⁵	8.5×10 ⁴	1.0×10 ⁴	1.2×10 ³	5.4×10 ³	2.8×10 ⁵
	5.8×10 ⁴	6.2×10 ⁴	1.0×10 ⁴	2.5×10 ³	4.8×10 ³	1.6×10 ⁵
	4.3×10 ⁴	4.6×10 ⁵	2.8×10 ⁴	6.9×10 ³	3.0×10 ³	8.9×10 ⁴
	5.0×10 ⁴	7.2×10 ⁴	3.1×10 ⁴	2.1×10 ³	2.2×10 ³	1.1×10 ⁵
	7.3×10 ⁴	7.2×10 ⁴	1.1×10 ⁴	1.8×10 ³	3.2×10 ³	1.2×10 ⁵
		6.4×10 ⁴				
		7.2×10 ⁴				
$\bar{x} \pm SD$	7.5±4.3×10 ⁴	6.7±4.7×10 ⁴	1.8±1.0×10 ⁴	2.9±2.3×10 ³	3.9±1.3×10 ³	1.5±0.7×10 ⁵
Student's "t"						
compared to		0.679	0.021	0.006	0.006	0.085
rubber P values						

Rubber and some plastic model devices contaminated with *Pseudomonas aeruginosa* were implanted subcutaneously into mice. After 5 days the c.f.u. values in the developed biofilm were determined

*Softened polyvinyl chloride

Results

Biofilm development on model devices. Using *P. aeruginosa* contamination (Table I) the highest number of bacteria was found on silicon rubber, somewhat, but not significantly lower was observed on rubber and polyethylene ($P=0.085$ and 0.679 respectively). The c.f.u. values were significantly reduced on teflon tubes (0.021) and a very low level of colonization was observed (P values= 0.006) on tygon and polypropylene (Table I).

In cases of *S. aureus* (Table II) the results were similar. The highest colonization was observed on rubber and silicon rubber ($P=0.390$), lower on polyethylene tubes not significantly ($P=0.137$), significantly lower levels was observed on teflon surface ($P<0.001$) and the lowest values were from on tygon and polypropylene ($P<0.001$, <0.001) (Table II).

Table II

Adherence of Staphylococcus aureus on rubber and plastic model devices implanted into mice

	c.f.u. values per mm ²					
	Rubber	Polyethylene	Teflon	Tygon*	Polypropylene	Silicon rubber
	1.0×10^5	3.7×10^5	3.6×10^4	9.7×10^3	6.4×10^3	1.3×10^5
	1.3×10^5	4.3×10^5	4.6×10^4	9.0×10^3	2.6×10^3	6.1×10^4
	1.4×10^5	2.0×10^5	1.1×10^4	3.0×10^3	4.3×10^3	9.4×10^4
	1.3×10^5	1.0×10^5	2.1×10^4	4.0×10^3	2.1×10^3	2.1×10^5
	1.2×10^5	2.2×10^5	2.1×10^4	1.1×10^3	1.1×10^3	4.1×10^5
$\bar{x} \pm SD$	$1.2 \pm 0.1 \times 10^5$	$2.3 \pm 1.4 \times 10^5$	$2.7 \pm 1.4 \times 10^4$	$5.4 \pm 3.8 \times 10^3$	$3.3 \pm 2.0 \times 10^3$	$1.8 \pm 1.4 \times 10^5$
Student's "t"						
compared to		0.137	<0.001	<0.001	<0.001	0.390
rubber P values						

Rubber and some plastic model devices contaminated with *Staphylococcus aureus* were implanted subcutaneously into mice. After 5 days the c.f.u. values in the developed biofilm were determined

*Softened polyvinyl chloride

The effect of the sterilization method on the biofilm production on rubber. Comparing the effect on biofilm development of UV and heat sterilization using rubber devices showed that with *P. aeruginosa* the levels of contamination ($P=0.023$) and the terminal c.f.u. values were significantly higher ($P=0.003$) after heat pre-treatment, (Table III).

In the case of *S. aureus* contamination experiments a similar trend was observed, without significant changes (Table IV).

Table III

Adherence of Pseudomonas aeruginosa to rubber implants sterilized by UV-irradiation or by heat

c.f.u. values per mm ²				
Rubber sterilized by UV		Rubber sterilized by heat		
initial c.f.u.'s	terminal c.f.u.'s	initial c.f.u.'s	terminal c.f.u.'s	
A	B	C	D	
1.0×10 ⁴	7.1×10 ⁴	7.6×10 ⁴	1.0×10 ⁵	
2.4×10 ⁴	5.8×10 ⁴	9.5×10 ⁴	1.0×10 ⁵	
6.1×10 ⁴	4.3×10 ⁴	7.1×10 ⁴	8.9×10 ⁴	
7.3×10 ⁴	5.0×10 ⁴	6.3×10 ⁴	1.4×10 ⁵	
3.4×10 ⁴	7.3×10 ⁴	7.5×10 ⁴	1.5×10 ⁵	
$\bar{x} \pm SD$	4.0±2.6×10 ⁴	5.9±1.3×10 ⁴	7.6±1.2×10 ⁴	1.2±0.3×10 ⁵
Student's "t"	A-B=0.191	A-C=0.023	C-D=0.017	B-D=0.003
P values				

Rubber tubes contaminated with *Pseudomonas aeruginosa* were implanted subcutaneously into mice. The contamination level was also determined (initial c.f.u. values). After 5 days of implantation rubber devices were removed and the germ count (terminal c.f.u. values) was determined
NB. Around the rubber implants, especially around the heat sterilized ones a purulent reaction was observed

The effect of repeated usage on biofilm formation on rubber. As shown in Table V reusing the rubber device caused a significant increase both in the contamination level ($P=0.005$) and in the terminal development of the biofilm ($P<0.001$) with *P. aeruginosa*.

With *S. aureus* (Table VI) similar results were observed: both the levels of contamination ($P<0.001$) and the terminal c.f.u. values increased significantly ($P<0.001$) after resterilization and recontamination.

Discussion

In our previous series of experiments [2] a mouse model was elaborated to study the development of bacterial biofilm and its characteristics. The biofilm produced on the inner and/or outer surface of medical devices is a nidus of chronic or relapsing infections. This well-known risk of modern medicine presents several problems. To

decrease the possibility of colonization a biocid (isothiazolones) may be incorporated in the plastic polymer [3] but this is applicable only for short-term uses of devices. Often, the replacement of the contaminated device is the only possibility.

Table IV

Adherence of Staphylococcus aureus to rubber implants sterilized by UV-irradiation or by heat

c.f.u. values per mm ²				
Rubber sterilized by UV		Rubber sterilized by heat		
initial c.f.u.'s	terminal c.f.u.'s	initial c.f.u.'s	terminal c.f.u.'s	
A	B	C	D	
8.9×10 ³	1.0×10 ⁵	5.5×10 ⁴	8.1×10 ⁴	
3.6×10 ⁴	1.3×10 ⁵	1.5×10 ⁴	1.5×10 ⁵	
7.2×10 ⁴	1.4×10 ⁵	7.1×10 ⁴	4.1×10 ⁵	
2.7×10 ⁴	1.3×10 ⁵	5.8×10 ⁴	9.7×10 ⁵	
3.6×10 ⁴	1.4×10 ⁵	1.3×10 ⁴	4.1×10 ⁵	
$\bar{x} \pm SD$	3.6±2.3×10 ⁴	1.3±0.2×10 ⁵	4.2±2.7×10 ⁴	4.0±3.5×10 ⁵
Student's "t"	A-B=0.008	A-C=0.683	C-D=0.049	B-D=0.115
P values				

Rubber tubes contaminated with *Staphylococcus aureus* were implanted subcutaneously into mice. The contamination level was also determined (initial c.f.u. values). After 5 days of implantation the rubber devices were removed and the germ count of the developed biofilm was determined (terminal c.f.u. values).

NB. Especially in the case of heat-sterilized rubber implants a purulent reaction was observed

Costerton et al. [1] described that the efficiency of biofilm development is different on various plastic materials. Our aim with the presented results was to develop an experimental model to study this problem. All the results were compared to those obtained with rubber, i.e. the classic material before the "plastic era".

From the presented results it is clear the e.g. tygon (softened polyvinyl chloride) gives about 2 log₁₀ exponent lower adherence than e.g. polyethylene. This may point a way, choosing special plastic materials to lowering the risk in using medical devices. It is also remarkable that the same trend in the biofilm producing effectivity observed both with *P. aeruginosa* and *S. aureus*, with quite different envelope structures, metabolic activity, etc. This fact may suggest that the bacterial adherence on devices may depend on simple physical characters, as e.g. smoothness, or hydrophobicity supposed by Fletcher and Loeb [4] studying marine pseudomonad attachment. The presented method seems to be suitable for testing other materials, as Vergères and Blaser [5] showed the adherence on glass beads, or the biofilm development may observable e.g. in the peritoneal cavity [6], and also there is a possibility testing the

effectivity of antibacterial pretreatments, as made by Liedberg and Lundberg [7] using silver coated urinary catheter pieces.

Table V

The effect of repeated heat sterilization of rubber implants contaminated with Pseudomonas aeruginosa on the development of biofilm

	c.f.u. values per mm ²			
	Heat-sterilized rubber		Repeatedly heat-sterilized rubber	
	initial c.f.u.'s A	terminal c.f.u.'s B	initial c.f.u.'s C	terminal c.f.u.'s D
	3.1×10 ⁴	5.0×10 ⁴	6.2×10 ⁴	3.8×10 ⁵
	8.9×10 ³	6.2×10 ⁴	3.1×10 ⁴	4.0×10 ⁵
	1.7×10 ⁴	8.0×10 ⁴	4.1×10 ⁴	3.2×10 ⁵
	2.2×10 ⁴	6.6×10 ⁴	4.9×10 ⁴	3.1×10 ⁵
	9.7×10 ³	7.1×10 ⁴	3.6×10 ⁴	2.1×10 ⁵
$\bar{x} \pm \text{SD}$	1.7±0.9×10 ⁴	6.6±1.1×10 ⁴	4.4±1.2×10 ⁴	3.2±0.7×10 ⁵
Student's "t"	A-B=<0.001	A-C=0.005	C-D=<0.001	B-D=<0.001
P values				

Heat-sterilized (100 °C, 30 min) rubber tubes contaminated with *Pseudomonas aeruginosa* (initial c.f.u. values) were implanted subcutaneously into mice. After 5 days the implants were removed, terminal c.f.u. values were determined (on 5 implants), others were resterilized, recontaminated (second initial c.f.u. values), and reimplanted for a further 5 days. At the end the second terminal c.f.u. values were determined.

NB. A very well pronounced and diffuse pyogenic reaction was observed especially around the repeatedly applied rubber implants

Experiments carried out with rubber have shown that plastic devices have not only the advantage of being disposable, guaranteed sterile materials, but the traditional use of rubber devices, resterilizing them on several occasions is enhancing the risk. Correct sterilization kills the bacteria, but do not destroys the polysaccharide matrix of the biofilm and in case of a new contamination the biofilm develops more rapidly [1]. On the other hand, on the basis of comparison UV and heat sterilization, the heat-treatment in itself causes such a change on the rubber surface that the level of the bacterial adherence is increasing.

Table VI

The effect of repeated heat sterilization of rubber implants contaminated with *Staphylococcus aureus* on the biofilm development

	c.f.u. values per mm ²			
	Heat-sterilized rubber		Repeatedly heat-sterilized rubber	
	initial c.f.u.'s	terminal c.f.u.'s	initial c.f.u.'s	terminal c.f.u.'s
	A	B	C	D
	3.5×10 ⁴	3.2×10 ⁵	8.9×10 ⁴	1.5×10 ⁶
	2.9×10 ⁴	4.0×10 ⁵	7.8×10 ⁴	1.4×10 ⁶
	4.5×10 ⁴	4.9×10 ⁵	9.5×10 ⁴	1.5×10 ⁶
	1.5×10 ⁴	4.9×10 ⁵	1.0×10 ⁵	1.7×10 ⁶
	4.4×10 ⁴	8.1×10 ⁵	8.2×10 ⁴	1.9×10 ⁶
$\bar{x} \pm \text{SD}$	3.4±1.2×10 ⁴	5.0±1.8×10 ⁵	8.8±0.9×10 ⁴	1.6±0.2×10 ⁶
Student's "t"	A-B=<0.001	A-C=<0.001	C-D=<0.001	B-D=<0.001
P values				

Heat-sterilized (100 °C, 30 min.) rubber tubes contaminated with *Staphylococcus aureus* (initial c.f.u. values) were implanted subcutaneously into mice. After 5 days implants were removed, the terminal c.f.u. values were determined using some of them. Others were re-sterilized and re-contaminated (second initial c.f.u. values) and reimplanted for further 5 days into mice. At the end these also removed and the terminal germ count was determined.

NB. A very well pronounced and diffuse pyogenic reaction was observable especially around the repeatedly applied rubber implants

Acknowledgements. This work was supported by the Scientific Research Council, Hungarian Ministry of Welfare (T559/1990) and by the Hungarian Research Fund (OTKA) grant 49.

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DEHYDROEPIANDROSTERONE MODULATES THE SPONTANEOUS AND IL-6 STIMULATED FIBRINOGEN PRODUCTION OF HUMAN HEPATOMA CELLS

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(Received September 19, 1994)

(Accepted October 19, 1994)

The role of an androgen, dehydroepiandrosterone (DHEA) has been studied on the constitutive and IL-6 induced fibrinogen production of HepG-2 cells. DHEA markedly augments the constitutive fibrinogen production of the hepatoma cells in a dose dependent fashion. Oppositely, for IL-6 induced fibrinogen production, DHEA is strongly inhibitory. The effectiveness of DHEA on the constitutive fibrinogen production is further potentiated if the hepatoma cells are preincubated with a glucocorticosteroid, dexamethasone. These findings demonstrate that the complex interaction between the steroid- and cytokine-directed regulation of the production of acute phase proteins is further coloured by the action of androgens on immune and hormonal systems.

It has been established recently that an active and considerable cooperation exists between the endocrine and immune system. Immune responses alter endocrine function and endocrine activity modifies immunological activity [1]. The disturbance of the immunological–endocrinological homeostasis may result in several diseases for example inflammations, virus infections.

The interaction was examined mostly in relation of glucocorticoids [2, 3] and sex hormones [4, 5], however the role of an other adrenal secretory product, the dehydroepiandrosterone (DHEA), has been revealed only recently [6].

DHEA and its sulphate ester are circulating in peripheral blood in a high level, however their basic biological function remains to be elucidated more precisely. The role of DHEA-DHEA sulphate in several, non-endocrine diseases has been described earlier. Deterioration of immunologic functions depending on age, medication and

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other factors was found to be in correlation with DHEA-glucocorticoid ratio. Many of the diverse effects of DHEA appear to be opposite those of glucocorticoids.

It has been established recently that DHEA influences cytokine production, particularly that of TH1 and TH2 cells playing a central role in the positive and negative regulation of the immune response [7].

In our present work we studied the interaction of DHEA and one of the central inflammatory cytokine, interleukin (IL)-6 on the production of one acute phase protein, fibrinogen in a human hepatoma cell, HepG-2.

Materials and methods

Cell culture. HepG2 cells (3×10^5 cell/well, one ml cultures) were cultured in RPMI 1640 containing 10% fetal calf serum, 200 nM glutamine, penicillin and streptomycin. HepG2 cells were stimulated for 24 h by recombinant human IL-6 (Sigma) (30 or 100 U/ml), and/or by various amounts (10^{-9} , 10^{-7} , 10^{-5} M) of DHEA (The British Drug House Ltd., Poole). In combination experiments the cells were treated with IL-6 and DHEA simultaneously. The experiments were performed in 24 well plates (Greiner, Germany) at 37 °C, 5% CO₂. In some experiments the cells were pretreated with 2 µM dexamethasone (Sigma) for 24 h.

Detection of fibrinogen in the supernatants of HepG2 cells. Fibrinogen levels were measured by ELISA using unlabelled and peroxidase-labeled IgG fractions of goat anti-human fibrinogen (Atlantic Antibodies). Absorbance at 492 nm of the OPD enzyme substrate was measured. Sensitivity limit for fibrinogen was 10 ng.

Statistical analysis. To evaluate the results, the Student *t* test was used (Stat-program, Blackwell).

Results

Figure 1 shows the changes of fibrinogen production of dexamethasone untreated or pretreated HepG-2 cells after DHEA addition in concentration 10^{-9} , 10^{-7} and 10^{-5} M. The data suggest, that DHEA elevates fibrinogen synthesis in a concentration dependent manner and the rate of increase was significant in the two higher doses. Dexamethasone pretreatment further potentiated the stimulatory effect of DHEA on fibrinogen production. These elevations were significant in all three DHEA concentrations if compared to the corresponding values from experiments without adding dexamethasone.

Figure 2 shows experiments with combined IL-6 and DHEA treatment using suboptimal (30 U/ml) or optimal (100 U/ml) IL-6 stimulation. Our findings suggest that DHEA suppresses the IL-6 stimulated fibrinogen synthesis, particularly when higher doses of DHEA were tested. Even if the tendency is similar, the effect is less significant in cases of stimulation with greater (optimal) amount (100 U/ml) of IL-6 (Fig. 3). In these experiments dexamethasone (which is strongly inhibitory alone) did not affect the inhibitory action of DHEA on fibrinogen production.

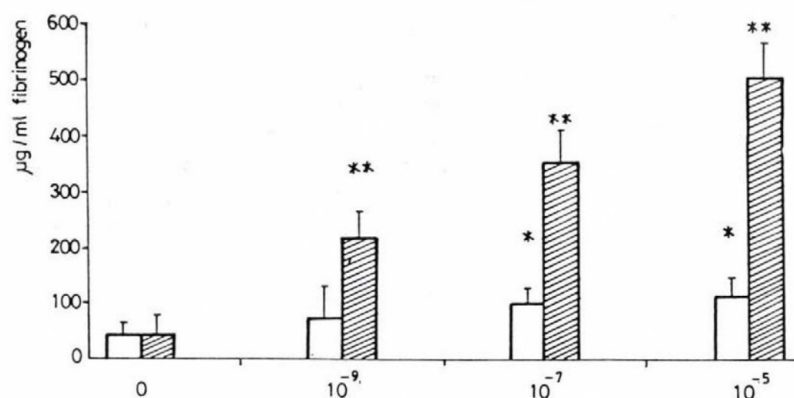


Fig. 1. The in vitro effect of DHEA on the constitutive fibrinogen production of HepG2 cells, in the absence or presence of 2 µM dexamethasone. The treatment of cells and the determination of fibrinogen is described in Materials and methods. Arithmetic mean and s.e.m. values are shown. Shaded columns: 2 µM dexamethasone pretreatment for 24 h; n=6. * = p<0.05, ** = p<0.01 (Student *t* test)

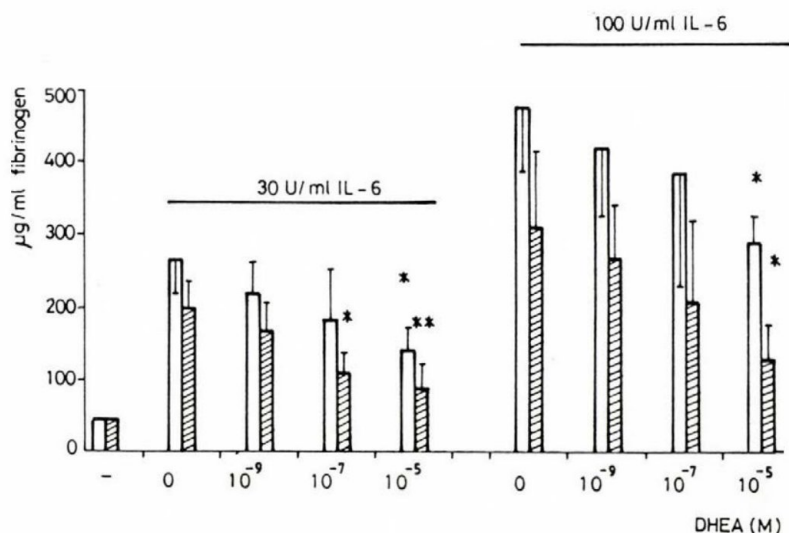


Fig. 2. The in vitro effect of DHEA on the fibrinogen production evoked by 30 U/ml IL-6 or 100 U/ml of HepG2 cells, in the absence or presence of 2 µM dexamethasone. The treatment of cells and the determination of fibrinogen is described in Materials and methods. Arithmetic mean and s.e.m. values are shown. P values (Student *t* test) were calculated comparing the corresponding values treated with 30 or 100 U/ml and those treated with IL-6 and DHEA. Shaded columns: 2 µM dexamethasone pretreatment for 24 h; n=6 (30 U/ml), n=4 (100 U/ml) * = p<0.05, ** = p<0.01 (Student *t* test)

Discussion

Growing interest has been devoted recently to the complex role in immunological processes of the adrenal androgens, DHEA. In our earlier studies we described some autoimmune diseases as "DHEA deficiency syndromes" [8–10].

In vitro and animal experiments DHEA effect was proved on the activation of complement system [11, 12] and also on the cytokine production [6, 7, 13]. It was found that DHEA affects on IL-2, IL-4, IL-5 and IFN γ synthesis of T-cells. While glucocorticoids reduced IL-2 and elevated IL-4 levels both in vivo and in vitro, oppositely, DHEA treatment of normal or glucocorticoid treated animals resulted in an enhanced capacity of their T-cells to produce IL-2.

In our present work the role of DHEA has been examined in the spontaneous and IL-6 stimulated fibrinogen expression of HepG-2 cell lines, as a model system for the production of acute phase proteins.

Our present data show that when acting alone, both DHEA and IL-6 concentration-dependently enhances the in vitro fibrinogen synthesis. However, when IL-6 and DHEA are combined they seem to behave as antagonists in this process. Further studies are in progress to check the possible role of this androgen in the regulation of IL-6 receptor expression.

The trends of stimulatory effect of DHEA on the constitutive fibrinogen production was more emphasized after pretreating the cells with dexamethasone. It is well documented that the number of IL-6 (and other cytokine) receptors is increased after a treatment with glucocorticosteroids [14]. Moreover, as an other regulatory effect, certain cytokines (such as IL-6, TNF α and IL-1) [15] elevate the number of glucocorticoid receptors. Our present data on the enhancement of DHEA action after dexamethasone pretreatment provokes a speculation that even the number (or availability) of putative "DHEA receptors" can be increased by glucocorticosteroids. Of course the confirmation of this idea needs further experimentation.

These preliminary data were obtained using DHEA in a concentration range corresponding to its physiological concentration in human. This fact further prompts studies in order to elucidate some cellular and molecular details of a putative complex regulatory network of androgens, glucocorticosteroids and inflammatory cytokines.

Acknowledgement. The authors are grateful to Krisztina F. Nagy for the excellent technical assistance.

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DETECTION OF HEPATITIS C VIRUS (HCV) BY REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

(A NOTE)

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(Received September 19, 1994)

(Accepted October 19, 1994)

Since the majority of post-transfusion hepatitis cases are closely related to the HCV infections, the sensitive and specific detection of HCV-RNA is a central issue both in blood transfusion and in follow-up of hepatitis patients. This leaflet provides the technical details of HCV-RT-PCR.

Isolation of RNA from serum. Sterile serum samples (20–40 µl) are diluted with a lysis buffer (0.2 M Tris-HCl, pH=7.5, 25 mM EDTA, 0.3 M NaCl, 2% sodium dodecylsulphate) containing 200 µg/ml Proteinase K. After 40 min incubation at 37 °C the nucleic acid is extracted from the samples by a single phenol-chloroform (24:1) extraction [1]. The aqueous phase is then precipitated by 96% ethanol and redissolved in RN-ase free distilled water.

RT-PCR. For the reverse transcription (RT) and the PCR the Perkin-Elmer mRNA-PCR kit was used. After RT with random hexanucleotides the amplification of cDNA was performed using two oligonucleotid primers (Fig. 1A) from the 5' end of the HCV genom [2] in the presence of TaqI polymerase. After 35 cycles (95 °C 1 minute, 60 °C 1 minute), the aliquots were electrophoretised on 4% SeaKem agarose. After staining with 0.01% ethidium bromide, the presence (or absence) of a specific 216 base pair (bp) long fragment corresponding to the sequence between –313 and –98 bp of HCV cDNA (Fig. 1B) has been checked. The kit's internal control in addition to positive and negative serum samples were used as control.

This method provides a reliable and quick test for checking the presence of hepatitis C virus.

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antisense: TTGCGGGGGGCACGCCCAA (18 bp)

sense:

CCATGAATCACTCCCCTGTGAGGAACTA (28 bp)

Fig. 1A. The nucleotide sequence of two primers for HCV RT-PCR (5'-3')



Fig. 1B. HCV-RNA positive and negative samples. The 216 bp bands indicate the presence of virus RNA. The internal standard is labelled by *

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Acta Microbiologica et Immunologica Hungarica publishes reviews and original papers on microbiology and immunology in English

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CONTENTS

Immunological Responses to Phospholipase-A of <i>Salmonella typhi</i> Punj, V., Hittu Matta, Kalra, M. S.	237
An Adenovirus-Herpes Simplex Virus Glycoprotein B Recombinant (Ad-HSV.gB) Protects Mice against a Vaccinia HSV.gB and HSV Challenge Endresz, V., Berencsi, K., Gönczöl, E.	247
Comparison of Petrifilm™ Method to Conventional Methods for Enumerating Aerobic Bacteria, Coliforms, <i>Escherichia coli</i> and Yeasts and Molds in Foods Jordano, R., Lopez, C., Rodriguez, V., Cordoba, G., Medina, L. M., Barrios, J.	255
Interaction of Immunomodulant Substances in Mouse Experiments with Special Regard to Drug Sensitivity and Bacterial Translocation Anderlik, P., Szeri, I., Antmann, K., Bános, Zs.	261
The Number of Glucocorticoid Receptors in Peripheral Human Lymphocytes is Elevated by a Zinc Containing Trace Element Preparation Falus, A., Béres, J. Jr.	271
Interaction, between Tricyclic Psychopharmacons and Some Antibiotics Molnár, J., Bathó, N., Csík, V., Chevalier, J., Cremieux, A.	277
Resistance to Beta-Lactam Antibiotics and Beta-Lactamase Production of <i>Bacteroides</i> , <i>Porphyromonas</i> and <i>Prevotella</i> Strains Nagy, E., Szőke, I., Gacs, M., Csiszár, K.	287
Effects of Pentoxifyllin and Pentaglobin ^o on TNF and IL-6 Production in Septic Patients Mándi, Y., Farkas, Gy., Ocsóvszky, I.	301
The Entire <i>rne</i> Gene: The Remaining Questions Miczák, A.	309
Complement C4, Factor B and C3 Polymorphism in North India Kramer, J., Srivastava, L. M., Ferenczy, E., Füst, G.	315
Study on the Glycoprotein Nature of Chicken IFNS by Chemical Cleavage and Inhibition of Glycosylation Taródi, B., Pusztai, R., Solymosi, T., Béládi, I.	321

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IMMUNOLOGICAL RESPONSES TO PHOSPHOLIPASE-A OF *SALMONELLA TYPHI*

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(Received September 2, 1994)

(Accepted November 10, 1994)

A 39 kD phospholipase-A was purified from *Salmonella typhi*. The enzyme was efficiently concentrated by precipitation with ammonium sulphate and purified by sequential use of column chromatography on DEAE-Sephacel and Sephadex G-100. Humoral and cellular immune responses were determined against both crude and purified phospholipase-A. Crude fraction was found to be more immunogenic than purified fraction.

Salmonella typhi is the etiological agent responsible for clinical typhoid fever, which remains one of the major public health problem in developing countries. Though immunogenicity of various components of *S. typhi* viz. H and O antigens [1], Vi [2] and porins [3] have been documented, yet there is a great paucity of information about microbial factors that determine pathogenicity and those that elicit immune responses in human beings with typhoid infections. Little attention has been given to immune reactions induced by enzymes of *S. typhi*.

Phospholipase-A metabolizes the membrane phospholipids to lysophospholipids consequently resulting in abnormal functioning of membrane and impairs the binding of immunoglobulin to membrane receptors [4]. Since these phospholipases affect the vital membrane functions of cells, it is expected that the phospholipase-A may have vital role in immunoprophylaxis of enteric fever. However, literature reveals only sparse information regarding immunological characterization of phospholipase-A of *S. typhi* [5–7]. Though Chau and Tsang [9] have reported immunogenicity of two proteins in *S. typhi* filtrates, but still the relative importance of either immune responses (humoral/cellular) and precise immunoprophylactic agents remain largely unclear. Hence the present investigation was undertaken to study the immune responses elicited by phospholipase-A fractions of *S. typhi* in relation to pathogenesis of disease in mice.

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Materials and methods

Bacterial strain. *S. typhi* S-799-88 obtained from the Central Research Institute, Kasauli, India, was maintained on brain heart infusion agar (BHI, Hi-media, Bombay, India).

Animals. Swiss albino mice (outbred) of either sex weighing 18–20 g (4–5 weeks old) were used throughout the study and fed on a standard pellet diet (Hindustan Lever Ltd., Bombay, India) with water ad libitum.

Preparation of antigen. A 24 h old growth of *S. typhi* in BHI broth was harvested by centrifugation at 10 000 g for 15 min and enzyme was isolated by ammonium sulphate precipitation (70% saturation). The precipitates were kept at 4 °C overnight, dissolved in the minimum amount of 0.05 M Tris HCl, pH 7.5 and then dialysed against the same buffer for 2 days in cold to ensure complete removal of ammonium sulphate. This was denoted as crude phospholipase-A (CP).

CP was further purified by sequential use of ion exchange chromatography on DEAE-Sephacel followed by gel filtration chromatography on Sephadex G-100, as explained previously [5]. The purified fraction was designated as purified phospholipase-A (PP). The homogeneity of the purified protein was monitored by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions [10]. Protein estimation at each stage was done according to the method of Lowry et al. [11].

Assay of enzyme activity. Enzyme activity was assayed by the method of Rao and Subrahmanyam [12] using phosphatidyl choline as substrate. One unit of enzyme activity was defined as µg of lysophosphatidyl choline formed per mg protein of enzyme.

Immunization schedule. Immunization of mice was carried out in groups of 20–30 animals with CP and PP. Group Ia received intraperitoneally a total amount of 200 µg of protein emulsified in Freund's complete adjuvant (FCA). Immunization was distributed in three injections on day 0, 5 and 10. Group Ib was immunized with a single intraperitoneal injection of CP, 200 µg in 0.1 ml emulsified in FCA. Group II received 150 µg of PP emulsified in FCA in three equally divided doses on day 0, 5 and 10.

With each group a control of animals (10 mice) was injected in parallel with saline emulsified with adjuvant in a corresponding schedule.

Measure of immune responses. The animals were bled before immunization and preimmunized serum was stored at –20 °C. From each group, 4 or 5 mice were bled to death and their spleens were removed aseptically on 1st, 2nd, 3rd and 4th week after complete immunization. The sera were separated and stored at –20 °C to check immunity by indirect haemagglutination (IHA) as described by Herbert [13]. Briefly, 10 ml of 2.5% SRBC (sheep red blood cells) suspension in phosphate buffer saline (pH 7.2) and 1:20 000 dilution of tannic acid in saline were mixed and incubated in a water bath at 37 °C for 30 min. The suspension was then centrifuged at 1200 g for 5 min and supernatant was discarded. Sedimented SRBC were washed thrice with phosphate buffer saline (pH 7.2) and suspended in same. This is designated as tanned SRBC. One ml of tanned SRBC and 1:2 dilution of antigen in phosphate buffer saline (pH 6.4) were mixed and incubated at room temperature for 2 h. The suspension was then centrifuged at 1200 g for 5 min. These cells were washed twice with normal saline, resuspended in normal saline and labeled as antigen sensitized SRBC. These SRBC were used to study agglutination with serial dilutions of inactivated test serum sample (56 °C for 30 min).

Aseptically removed spleens were used to study cell mediated immune responses employing leukocyte migration inhibition (LMI) technique of Falk and Zabriskie [14]. The standardized concentrations of various antigens viz. CP (75 µg/ml) and PP (100 µg/ml) were used to avoid false positive results of the test.

Statistical analysis. Results were presented as arithmetic ± standard deviation.

Results

Purification. The chromatographic pattern of CP obtained after ammonium sulphate precipitation on DEAE-Sephacel showed that enzyme activity was eluted in fractions from 31 to 58 predominately of 0.2 M potassium chloride gradient (Fig. 1). These fractions were pooled and concentrated for further purification. This step led to 35.57-fold increase in specific activity with 6.9% protein yield (Table I). The pooled and concentrated fractions were chromatographed on Sephadex G-100. The elution profile showed two distinct peaks (Fig. 2). All the enzyme activity was observed in peak II, hence fractions No. 25 to 40 were pooled, concentrated, lyophilized and designated as PP. This three step purification led to 58.07-fold purification of the enzyme. The enzyme was found to be homogeneous having molecular weight of 39 kD on SDS-PAGE (Fig. 3).

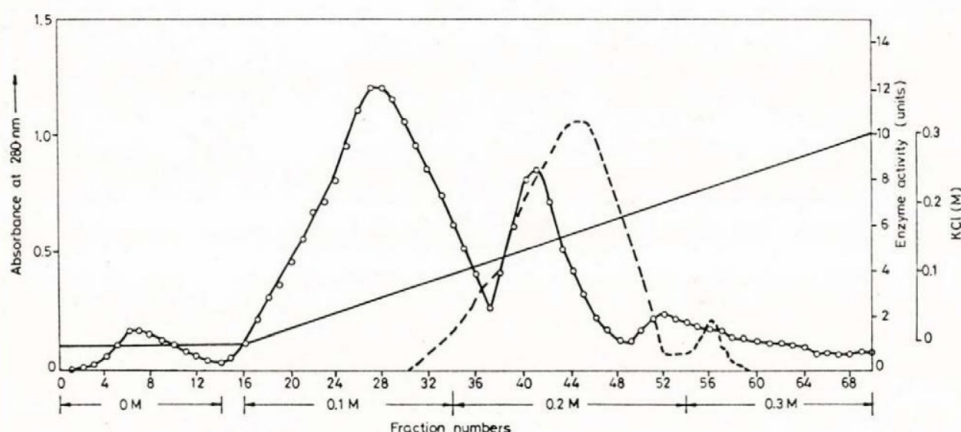


Fig. 1. Elution profile of phospholipids A on DEAE sephacel column chromatography absorbance at 280 nm (O—O); enzyme activity (-----); KCl gradient (——). 1 unit = 1 μ g of LPC formed per mg of protein

Immune responses with CP. Agglutinating antibodies determined by the IHA test showed an increase in geometric mean titre from 256 in the first week after immunization to a maximum of 3072 on 3rd post immunization week. A single dose immunization procedure induced a relatively weak immune response and a measurable agglutinating antibody titre could not be detected in animals up to two weeks post immunization (Fig. 4).

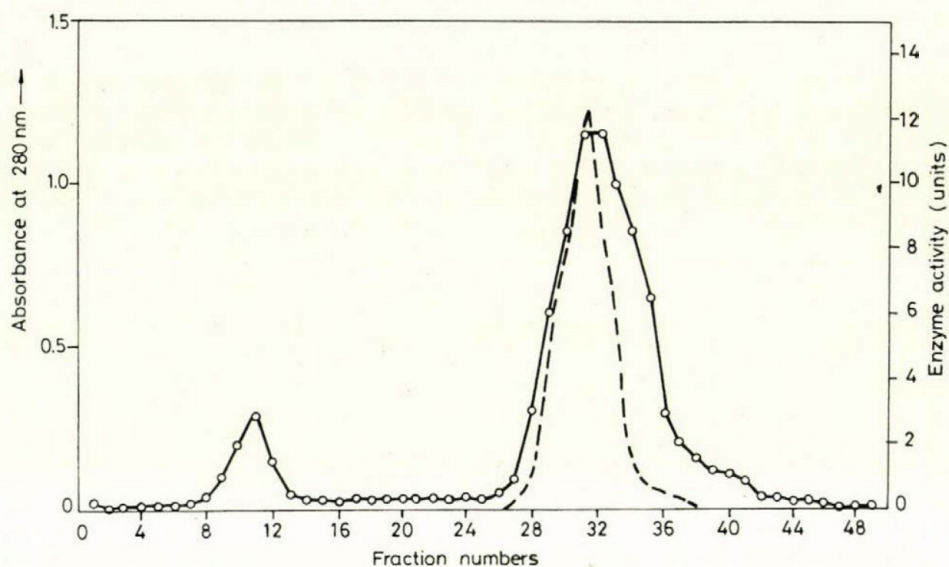


Fig. 2. Elution profile of phospholipase A enzyme active fractions from DEAE sephacel column on sephadex G-100; absorbance at 280 nm (O—O); enzyme activity (-----)

Table I

Purification of phospholipase-A of S. typhi

Purification	Total protein (mg)	Total activity (units)	Specific activity (units)	Purification (fold)	Yield (%)
Cell free supernatant	2000.00	940.00	00.52	1.00	100.00
Ammonium sulphate precipitation (70%)	500.00	510.32	3.36	6.46	54.28
DEAE-Sephacel	16.34	95.39	18.50	35.57	10.14
Sephadex G - 100	3.06	13.40	30.20	58.07	1.42

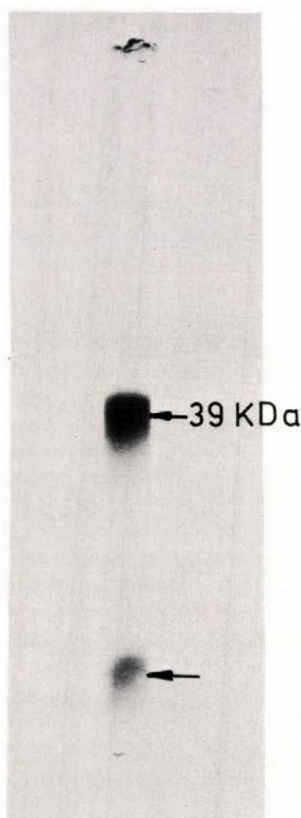


Fig. 3. Disc polyacrylamide gel electrophoretic pattern of purified phospholipase-A of *S. typhi*.
(← : movement of dye)

The cellular immune responses evaluated by LMI showed significant cellular sensitization (Fig. 5). Migration inhibition less than 20% was considered as non significant. Significant migration in immunization with single dose injection could only be observed after the second week of immunization, however, multiple injections schedule produced a significant ($p < 0.2$) migration inhibition after one week of immunization.

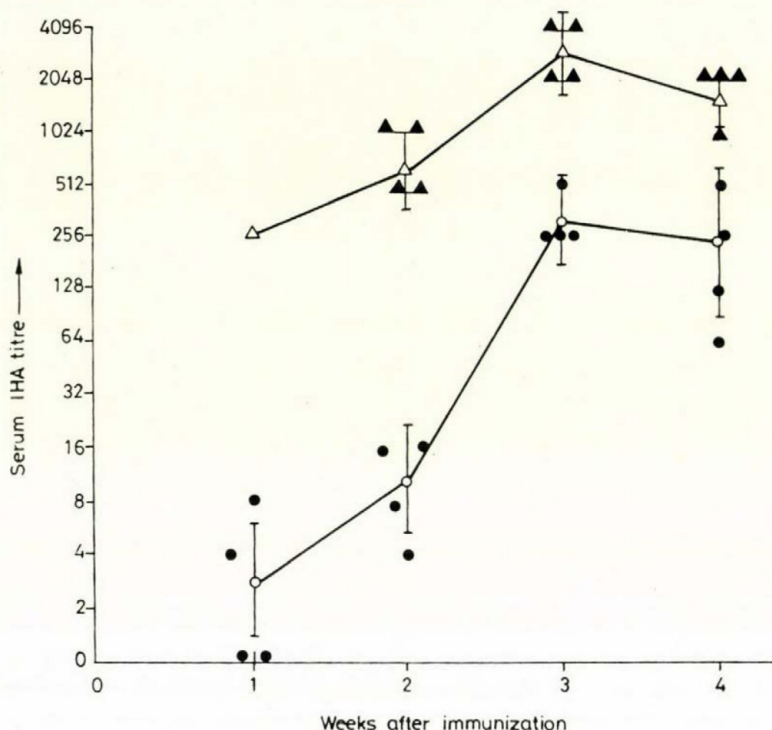


Fig. 4. Indirect haemagglutinating titre in mice immunized with single (O—O) and multiple (Δ—Δ) doses of CP of *S. typhi*. Vertical bars represents standard deviation (2SD) of means

Immune responses by PP. Since multiple injection schedule was found to be better as compared to single injection schedule, only multiple schedule was followed with PP. From Fig.6 it is evident that the antibody titres did not differ significantly on the 1st and 2nd week after immunization. However, a significant increase ($p < 0.05$) in antibody titre was obtained on 3rd post immunization week (1:2560). The antibody titre remained almost same on the 4th week.

The maximum LMI of $56.25 \pm 12.38\%$ was observed on the 2nd week after immunization. The control mice did not show any cell mediated immune responses.

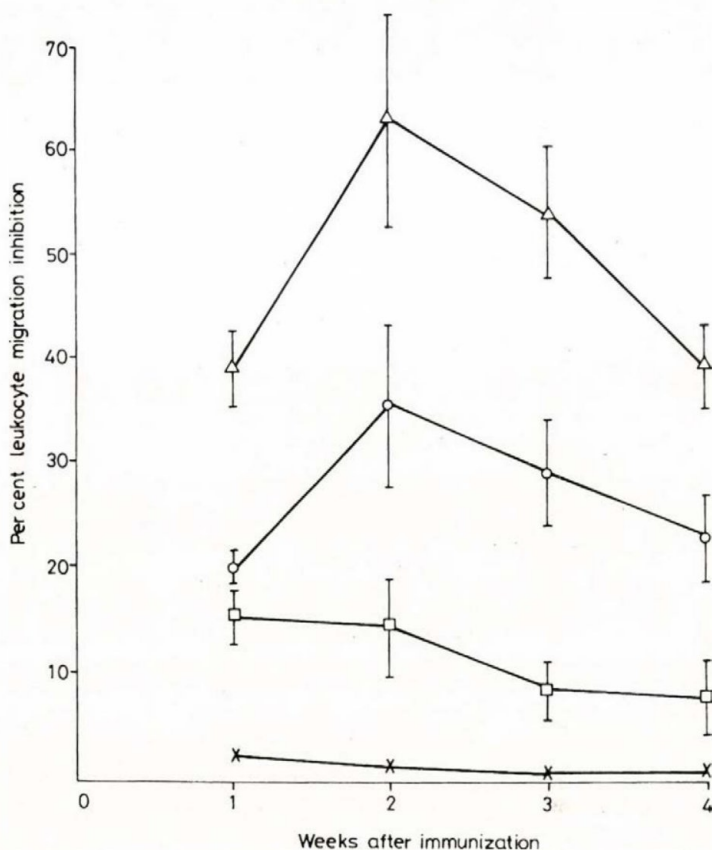


Fig. 5. Leukocyte migration inhibition in mice immunized with single (○—○) or multiple (Δ—Δ) doses of CP of *S. typhi*. Control (□—□), preimmunized serum (×—×). Vertical bars represent standard deviation (2SD) of means

Discussion

Phospholipase-A has been extensively studied in relation to stimulus linked liberation of fatty acid from membrane phospholipids, which might regulate transport across membrane and permeability of the membrane towards macromolecules thus they could participate in some vital membrane functions such as growth and fusion [4, 15], which may alter the immunopathogenesis of disease.

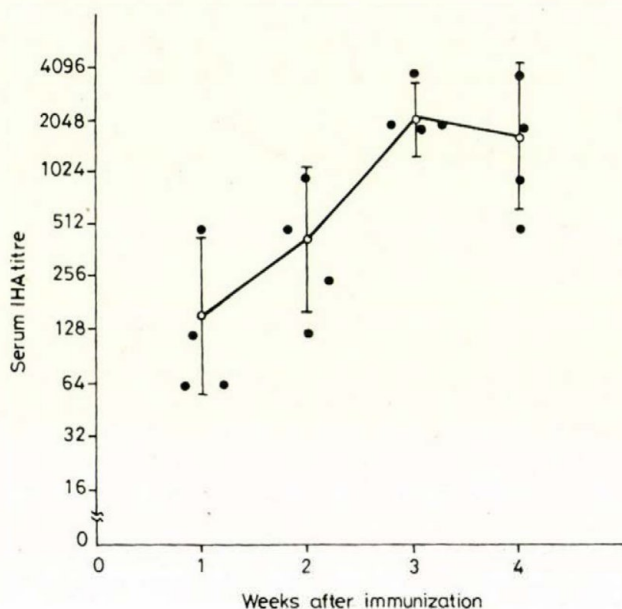


Fig. 6. Indirect haemagglutinating antibody titre in mice immunized with PP of *S. typhi*. Vertical bars represents standard deviation (2SD) of means

As a first step towards understanding of the immunoprophylaxis of enteric fever, a 39 kD phospholipase-A of *S. typhi* was efficiently purified by sequential use of ammonium sulphate precipitation, column chromatography on DEAE-Sephacel and Sephadex G-100. Using SDS solubilization, acetone-ethanol precipitation and PAGE, a membrane bound 29 kD phospholipase-A has been purified from *Escherichia coli* by Scandella and Kornberg [16]. While, 71 and 65 kD phospholipase-A have been purified from *Salmonella newport* [17] and *S. typhi* [6] respectively. The behaviour of phospholipase-A depends largely on occurrence and assay conditions. Moreover, the method of purification also influence the yield of purified enzyme [4].

Since the aim of present study was to select immunologically active protein with potential for use as immunoprophylactic agent, harsh physico-chemical treatments of the bacteria which may cause protein denaturation were avoided [18]. Both CP and PP elicited humoral as well as cellular immune responses. Humoral responses with either CP or PP reaching peaks after the 3rd post-immunization week, suggested that further boosting may be necessary to maintain the higher antibody titres. However, cellular immune responses declined after the 2nd post immunization week. This can be attributed to the differential regulation of immune responses in experimental salmonellosis [19, 20]. On comprehensive analysis of the immune responses in mice

immunized with either fraction of phospholipase-A, CP can act as better immunogen than its subfractions PP. This may be attributed to complex nature of said protein.

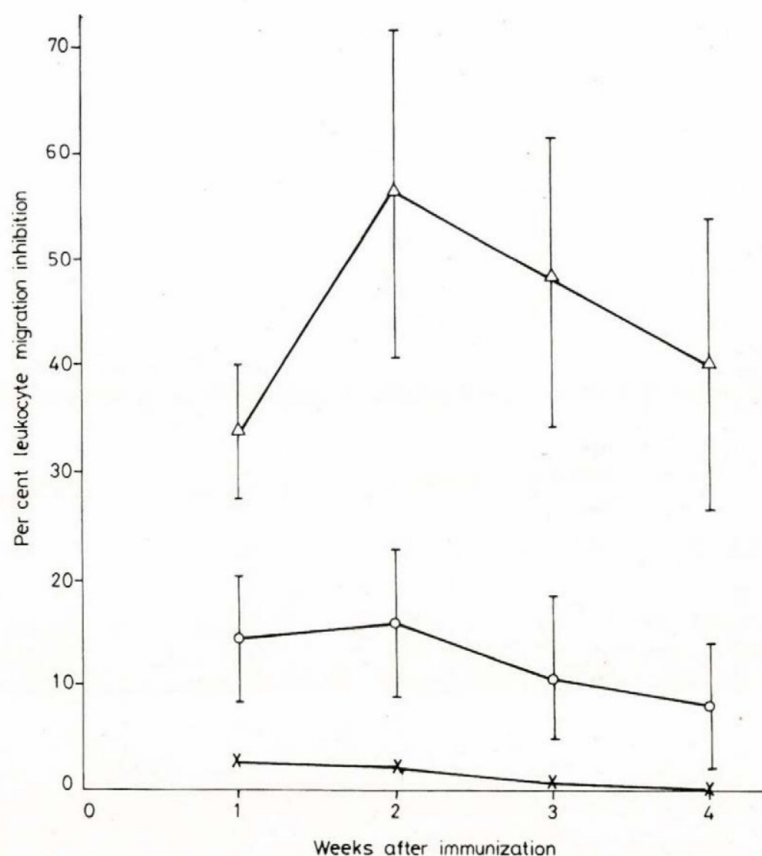


Fig. 7. Leukocyte migration inhibition in mice immunized with PP (Δ—Δ) of *S. typhi*. Control (O—O), preimmunized serum (x—x). Vertical bars represents standard deviation (2SD) of means

Thus we tend to feel that CP could act as a promising protective immunogen. One of our earlier report suggests that CP immunization of mice-with CP could induce a higher level of protection than PP against LD₅₀ of *S. typhi* [6]. In view of this, further studies on immunological basis of the protective mechanism mediated by phospholipase are needed to be explored in mucin mice model.

Acknowledgement. This work was supported by University Grants Commission, New Delhi, India.

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AN ADENOVIRUS-HERPES SIMPLEX VIRUS GLYCOPROTEIN B RECOMBINANT (Ad-HSV.gB) PROTECTS MICE AGAINST A VACCINIA HSV.gB AND HSV CHALLENGE*

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(Received September 27, 1994)

(Accepted December 30, 1994)

We investigated the mechanism by which mice immunized with an adenovirus-herpes simplex glycoprotein B recombinant (Ad-HSV.gB) are protected from challenge with a vaccinia (Vac)-HSV.gB and the cognate virus, HSV. C57/BL mice immunized intraperitoneally with Ad-HSV.gB were protected against an intracerebrally inoculated lethal dose of a Vac-HSV.gB or HSV-1. CD8⁺ cytotoxic T lymphocytes but not interferon- γ -dependent mechanisms play a major role in the clearance of both viruses from the central nervous system. These results demonstrate that the administration of two recombinant viruses carrying the same foreign insert for either immunization or challenge in a mouse protection model provides useful information about the protective nature of the inserted gene product.

Scientific strategies of modern vaccinology have only recently superseded the largely empirical approaches of the past and are now based on an understanding of immune response mechanisms, on identification of the relevant target antigens to elicit humoral and cellular immune responses, and on effective delivery systems to present the protective epitopes to immune cells. The availability of cloned proteins of a pathogen through recombinant DNA technology now allows analysis of their immunogenicity and of their protective ability. The latter studies are particularly important because an actual protective effect of an individual protein may depend on the time course of its expression in the infected host cell, and on its ability to induce a full complement of protective mechanisms.

Protection studies of individual proteins require an animal model for immunization and subsequent challenge with the pathogen. In the present study, we immunized mice with recombinant constructs of adenoviruses (Ad) carrying the herpes simplex virus (HSV) glycoprotein B gene (Ad-HSV.gB) [1] or Ad carrying a truncated

*This paper is dedicated to Professor Ilona Béládi, director emeritus of the Institute of Microbiology of Albert Szent-Györgyi University Medical School in Szeged, Hungary, by her pupils on the occasion of her 70th anniversary.

form of HSV-gB (Ad-HSV.gBd3) [2] and challenged them with a neurovirulent strain (WR) of Vac recombinant carrying the HSV-gB (Vac-HSV.gB) in order to analyze the protective mechanisms against Vac-HSV.gB. We have also compared the relative protective effect of HSV-gB against Vac-HSV.gB-recombinant and wild-type HSV infection.

Materials and methods

Cells. A549 human lung carcinoma cells (obtained from ATCC, Rockville, Md, USA) were used for propagation and titration of Ad viruses. VERO African green monkey kidney cells (from ATCC) were used for propagation and titration of Vac viruses. Cells were grown in minimal essential medium supplemented with 2 mM glutamine and 7.5% fetal calf serum (HyClone, Logan, Utah, USA).

Viruses. Adenovirus type 5 (Wt-Ad) was obtained from ATCC. Ad-HSV.gB, a recombinant Ad carrying the entire HSV-gB gene, and originally denoted as Ad-HSV.gB2 [1], and Ad-HSV.gBd3, an Ad recombinant with a mutated gB gene deleted in the 462-711 amino acid coding region [2], were both kindly provided by Dr. D. Johnson (McMaster University, Hamilton, Ontario, Canada). A Vac WR strain recombinant virus expressing the entire HSV-gB gene (Vac-HSV.gB) was obtained from Dr. B. Moss (National Institutes of Health, Bethesda, Md, USA). Ad and Vac viruses were purified by CsCl gradient and sucrose gradient centrifugation, respectively. Preparations of the HSV-1 strains, HFEM [3] and KOS-M [4], were provided by Dr. S. Deshmane (The Wistar Institute, Philadelphia, Pa, USA).

Mice and immunization. Female C57/BL mice and female C57/BL beige mice were purchased from Jackson Laboratories (Bar Harbor, Me, USA). Two breeding pairs of mice nullizygous for β_2 -microglobulin (constructed from C57BL/6 and 129 Ola mice, both of H-2^b haplotype) were obtained from Jackson Laboratories and bred in The Wistar Institute Animal Facility. These mice express little, if any, functional major histocompatibility complex (MHC) class I antigen on the surface and have a striking absence of CD8 cells [5, 6]. Mice were immunized intraperitoneally (i.p.) at age 6-7 weeks with 2×10^8 p.f.u. of purified Ad viruses or with 5×10^5 p.f.u. of HSV-1 KOS-M. For intracerebral (i.c.) challenge, 10 LD₅₀ of Vac-recombinant or HSV-1 HFEM were given in a 30- μ l volume.

Antibody. Anti-interferon (IFN)- γ antibody was inoculated i.p. 4 h before i.c. challenge (10^4 neutralizing units/2 mg IgG produced in rat) [7]. Control mice were treated with 2 mg of normal rat IgG (Sigma, St. Louis, Mo, USA).

Results

Protection of immunocompetent cytotoxic T lymphocyte (CTL) responder mice and beige mice with impaired natural killer (NK) cell function against a Vac-HSV.gB challenge after immunization with Ad-HSV.gB or Ad-HSV.gBd3 recombinant. As shown in Fig. 1, 90% of C57/BL mice immunized with 2×10^8 p.f.u. of Ad-HSV.gB and challenged i.c. 5 days later with 10 LD₅₀ of Vac-HSV.gB survived the Vac recombinant infection, whereas all non-immune mice and mice immunized with the Wt-Ad died after the Vac-HSV.gB challenge. These experiments demonstrate the HSV.gB specificity of protection after Ad-HSV.gB immunization. To test whether CTL, which play an important role against Vac virus infection, were responsible for the protective effects, C57/BL mice were immunized with the Ad-HSV.gBd3 recombinant virus, which does not induce a gB-specific CTL response in C57/BL mice due to a deletion in the gB coding region that contains the CTL epitope [2]; 30% of

mice survived the Vac-HSV.gB challenge (Fig. 1). C57/BL beige mice, which have impaired NK cell activity [8], were also immunized; survival in these mice was similar to that of the normal C57/BL mice, i.e., 90% of mice immunized with the CTL-inducer Ad-HSV.gB recombinant but only 40% of beige mice immunized with the CTL-non-inducer Ad-HSV.gBd3 recombinant survived the challenge. These data argue against the involvement of NK cells in the observed protection and indicate a correlation between the insert-specific CTL response induced by the Ad-recombinant and the protective effect of immunization. HSV-specific neutralizing antibodies are not responsible for the protection, since no HSV-neutralizing activity was detectable in the mouse sera 5 days after Ad-HSV.gB inoculation (not shown) and the inserted gene product is not a structural protein of Vac-recombinants [9].

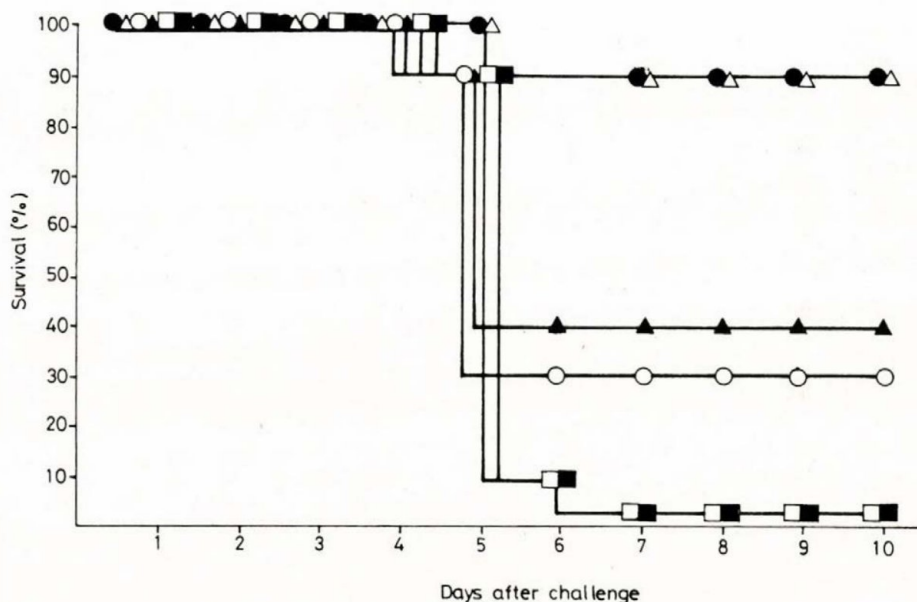


Fig. 1. Ten to 20 C57/BL normal or beige mice (H-2^b) of each group were inoculated i.p. with 2×10^8 p.f.u. of Ad viruses and challenged i.c. 5 days later with 10 LD₅₀ of a Vac-HSV.gB. Mice died at 4-8 days after challenge. ● normal mice, Ad-HSV.gB-immunized; ▲ beige mice, Ad-HSV.gB-immunized; ○ normal mice, Ad-HSV.gBd3-immunized; ■ beige mice, Ad-HSV.gBd3-immunized; □ normal mice, not immunized; ■ normal mice, Wt-Ad-immunized

Lack of protection of β_2 -microglobulin⁻, CD8⁺ T cell-deficient mice against a Vac-HSV.gB challenge after immunization with Ad-HSV.gB. To further analyze the correlation between protection and insert-specific CTL induction, a similar experiment was designed using C57/BLx129 β_{2m} -nullizygous mice and control C57/BL mice

immunized with Ad-HSV.gB (CTL-inducer) and challenged with Vac-HSV.gB 5 days later. As shown in Fig. 2, normal C57/BL mice were protected by immunization with the Ad-recombinant, whereas $\beta_{2m}^{-/-}$ mice, which have no functional MHC molecules and thus cannot present viral peptides for CTL recognition, did not survive the Vac-recombinant challenge, nor did control Wt-Ad-immunized normal and Wt-Ad-immune or non-immune $\beta_{2m}^{-/-}$ mice. These results confirmed that CTL induced by an Ad-HSV.gB-recombinant protect mice against a challenge infection with a Vac-HSV.gB-recombinant.

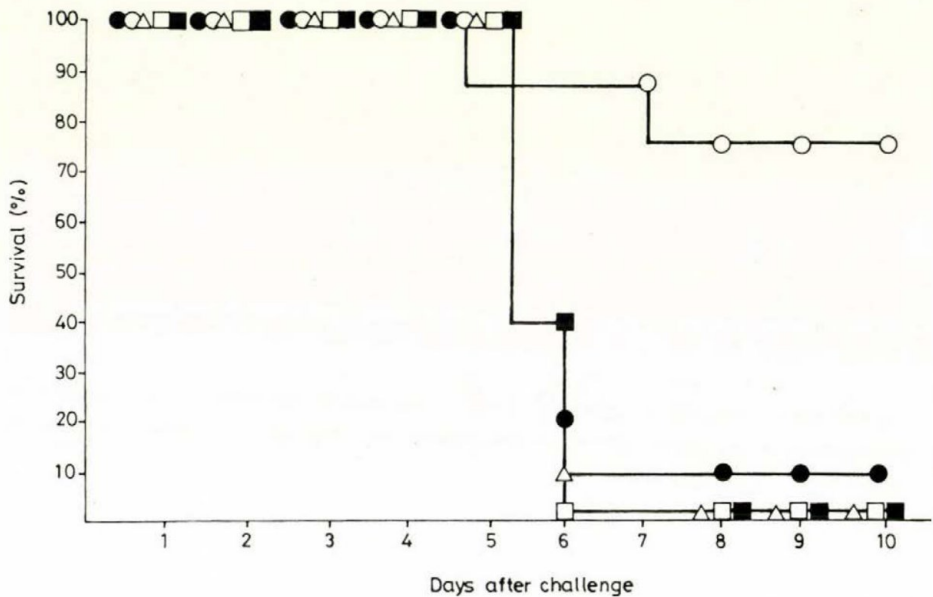


Fig. 2. Ten to 20 $\beta_{2m}^{-/-}$ mice (H-2^b background) and control C57/BL mice of each group were inoculated i.p. with 2×10^8 p.f.u. of Ad viruses and challenged i.c. 5 days later with 10 LD₅₀ of a Vac-HSV.gB. Mice died at 4–8 days after challenge. ○ normal mice, Ad-HSV.gB-immunized; △ $\beta_{2m}^{-/-}$ mice, Ad-HSV.gB-immunized; ● normal mice, Wt-Ad-immunized; □ $\beta_{2m}^{-/-}$ mice, Wt-Ad-immunized; ■ $\beta_{2m}^{-/-}$ mice, not immunized

Protection of immunocompetent, CTL-responder mice against Vac-HSV.gB correlates with protection against HSV-1; IFN- γ is not involved in the protection. The observation that Wt-Ad-immunized or non-immune mice died after the Vac-recombinant challenge, whereas 30–40% of Ad-HSV.gBd3 (CTL-non-inducer)-immunized mice survived the challenge (Fig. 1), i.e., the CTL-non-inducer virus induced a partial protection, suggested that mechanisms other than CTL induction contribute to the protective effect of the Ad-recombinant. Although the CTL epitope of HSV-gB is absent in the Ad-HSV.gBd3 recombinant, epitopes may still be present that

induce a helper T cell response, raising the possibility that the observed partial protection reflects the effect of IFN- γ produced by the helper T cells. To examine the role of IFN- γ in the protection, mice immunized with the Ad-HSV.gB were treated with anti-IFN- γ neutralizing antibody or control IgG and then challenged with Vac-HSV.gB; both anti-IFN- γ treated and control IgG-treated mice were similarly (90% survival) protected against Vac-recombinant challenge (Table I), ruling out any major role for IFN- γ in the protection after Ad-HSV.gB immunization. In a separate experiment the anti-IFN- γ antibody neutralized IFN- γ in vitro (not shown), indicating that the 90% survival of Ad-HSV.gB-immunized and anti-IFN- γ treated mice did not reflect the ineffectiveness of these antibodies.

Table I

IFN- γ independent, CD8 lymphocyte-mediated protection in Ad-HSV.gB-immunized, HSV.gB-specific CTL-responder C57/BL mice

Immunization	Treatment	Challenge	Survival (%)
Ad-HSV.gB	—	Vac-HSV.gB	90
Wt-Ad	—	Vac-HSV.gB	10
Ad-HSV.gB	—	HSV	85
Wt-Ad	—	HSV	20
Ad-HSV.gB	anti-IFN- γ	Vac-HSV.gB	90
Ad-HSV.gB	control IgG	Vac-HSV.gB	90
Ad-HSV.gB	anti-IFN- γ	HSV	80
Wt-Ad	control IgG	HSV	10

Ten-15 mice of each group were immunized i.p. with Ad-viruses and challenged i.c. with the Vac-HSV.gB or HSV-1 (10 LD₅₀) as described in the text. Anti-IFN- γ and control rat IgG were administered as described in the text

All of the experiments above tested protection induced by Ad-recombinants against an unrelated recombinant virus, i.e., Vac-recombinants. To determine whether protection against a Vac-recombinant parallels the protection against the cognate virus, we compared the protective immunity against Vac-HSV.gB and HSV-1 (Table I). We found that C57/BL mice immunized with 2×10^8 p.f.u. of Ad-HSV.gB were protected not only against i.c. challenge with Vac-HSV.gB, but also against i.c. challenge with HSV-1 strain HFEM. Anti-IFN- γ treatment did not affect the protection against HSV-1 infection. These data show that Ad-HSV.gB-immunized mice are protected against Vac-recombinant and HSV-1 infection, i.e., in this experimental setting, protection of Vac-recombinant infection correlates with that observed in HSV-1 infection. As controls, C57/BL mice were immunized i.p. with 5×10^5 p.f.u. HSV-1 strain KOS-M

and challenged 5 days later with an i.c. inoculation of HSV-1 HFEM or Vac-HSV.gB; all immunized mice survived, while mice not immunized died (not shown).

Discussion

Previous studies have demonstrated that influenza virus-immunized mice are protected against i.c. challenge with a Vac-influenza recombinant [10], and that CD8 or CD4, IFN- γ , and tumour necrosis factor- α -dependent responses are protective against a Vac-vesicular stomatitis (VSV) nucleoprotein (Vac-VSV-NP) recombinant virus in VSV-NP CTL-responder and CTL-nonresponder mice, respectively, after immunization with VSV [11, 12]. Our studies extend those observations by demonstrating that mice are protected against a challenge with a Vac recombinant even when the recombinant gene is expressed by a heterologous immunizing recombinant virus, such as Ad, carrying a homologous insert. Note, however, that in our Vac recombinant viruses as well as in the Vac-VSV-NP [11] used for the challenge studies, the inserted gene is under the control of an early and late Vac promoter. It remains to be shown whether replication of challenge Vac viruses with inserts under the late Vac promoter is inhibited by insert-specific immune mechanisms.

An important question is whether the protection demonstrated in our mouse model system indicates protection against the cognate virus. We observed that Ad-HSV.gB-immunized C57/BL mice were protected against a lethal challenge dose of HSV as well as Vac-HSV.gB and that anti-IFN- γ treatment of the mice did not influence the outcome of the challenge with either virus. However, Ad-HSV.gB-immunized $\beta_{2m}^{-/-}$ mice died after Vac-HSV.gB or HSV challenge, indicating that IFN- γ -independent, CD8⁺ T-cell mediated responses were involved in the HSV.gB-specific protection against both challenge viruses in the experimental conditions used. These data provide an example of correlated protection of Ad-recombinant (Ad-HSV.gB)-immunized mice against two challenge infections, a Vac-recombinant carrying the corresponding foreign gene (Vac-HSV.gB) and the natural host of the foreign gene (HSV).

Protection against a pathogen is a complex phenomenon, in which both humoral and cellular arms of the immune response exert their functions independently or in concert. Thus, it is difficult to study separately the protective role of the antibody and T cell responses to a particular protein. Protection by an Ad-HSV.gB recombinant virus against HSV challenge administered 28 days after the immunization in mice has been described [13], but the relative roles of humoral and cellular immunity were not determined in that model. Since the inserted gene products are not structural proteins of the vaccinia recombinants [9], our results provide evidence of protective T cell responses in the absence of HSV.gB-specific neutralizing antibody. Our comparative analysis of the protective effect of Ad-HSV.gB against a HSV-challenge and the protection against a Vac-HSV.gB showed that Ad-HSV.gB-immunized HSV.gB CTL-responder C57/BL mice were protected against both challenge viruses (90% survival in Vac-HSV.gB-challenged and 85% survival in HSV-challenged mice). Other groups of C57/BL mice immunized with Ad-HSV.gB and treated with anti-IFN- γ or control IgG

before challenge with Vac-HSV.gB or HSV were equally protected, suggesting that IFN- γ does not play a crucial role in the protection of CTL-responder mice against Vac-HSV.gB or HSV challenge. Other mechanisms, e.g., CD4⁺ T cell- and/or tumour necrosis factor- α -mediated responses might be involved in the 30% protection of mice immunized with the CTL-non-inducer Ad-HSV.gBd3 recombinant.

The availability of a mouse strain with an H-2^b background but lacking β_{2m} and thus all surface class I molecules, allowed us to assess the role of CD8⁺ T cell-mediated cytotoxicity protection. These mice were completely unprotected against Vac-HSV.gB challenge, demonstrating directly that CD8⁺ CTL are required for the clearance of Vac-HSV.gB from the central nervous system of CTL-responder mice. Although this mechanism of virus elimination requires that the virus-infected cell express class I MHC antigens, and neurons do not express these antigens, class I and II MHC antigens can be induced on other brain cells susceptible to Vac and HSV infection, such as meningeal cells, astrocytes, microglia, and oligodendrocytes [14–17]. HSV replicates in neuronal cells as well, and it has been suggested that the production of some soluble factors by CD8⁺ lymphocytes underlies the CD8-mediated clearance of HSV from the nervous system [18]. β_{2m} ^{-/-} mice inoculated intradermally with a high dose of a Vac WR strain completely recovered from the infection [19], indicating that the immune system provides sufficient overlap to protect β_{2m} ^{-/-} mice from the Vac-WR infection under these conditions. Our results obtained in the β_{2m} ^{-/-} mice indicate that the CD8⁺ T lymphocytes are the primary mediators of Vac virus protection in conditions of i.c. inoculation. Ad-HSV.gB-immune C57/BL beige mice were protected against Vac-HSV.gB challenge, confirming that the lack of NK activity does not prevent insert-specific vaccinia protection. In summary, our results indicate that in the absence of HSV-specific neutralizing antibodies there is a correlation in the mechanism of protection against a Vac recombinant (Vac-HSV.gB) and against the cognate virus (HSV), and that CD8 CTL play a major role in the protection against both viruses in the mouse brain.

Acknowledgements. We thank Joseph Marton for expert technical assistance and Marina Hoffman for editorial work. This study was supported by a grant from The Wistar Institute.

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COMPARISON OF PETRIFILM™ METHOD TO CONVENTIONAL METHODS FOR ENUMERATING AEROBIC BACTERIA, COLIFORMS, *ESCHERICHIA COLI* AND YEASTS AND MOLDS IN FOODS

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(Received October 11, 1994)

(Accepted November 23, 1994)

The Petrifilm™ plates method was compared to conventional methods (PCA, VRBA, Levine EMB agar and OGYE agar) for enumeration of mesophilic aerobic bacteria, coliforms, *Escherichia coli*, and yeasts + molds in six homogeneous lots of different food groups (pasteurized milk, yoghurt ice cream, eggs, minced meat, fresh strawberries and frozen green beans). For all the microbiological criteria except for yeasts and molds and mesophilic aerobic bacteria in frozen green beans, the mean values of counts with Petrifilm™ plates were higher than those obtained with traditional methods. The correlation coefficient of Petrifilm™ aerobic bacteria, coliforms, and yeasts + molds v. PCA, VRBA and OGYE agar for each microbiological criterion for a composite of six food products were 0.897, 0.861 and 0.981, respectively.

The Standard Methods for the Examination of Dairy Products (SMEDP), an American Public Health Association (APHA) compilation of microbiological methods for analyzing dairy foods and dairy food substitutes, in its 16th edition recognized the Petrifilm™ aerobic count (PAC) as an alternative method for standard plate count and the Petrifilm™ coliform count (PCC) for coliform organisms [1].

The Association of Official Analytical Chemists (AOAC) and the Association Française de Normalisation (AFNOR) have also validated this method for aerobic counts and coliforms. Vasavada and White [2] also include Petrifilm™ among the new methods introduced as alternatives or adjuncts to conventional methods in dairy microbiology.

Petrifilm™ plates have been used for determining total bacteria counts and coliforms in milk, dry milk powder, and ice cream [3]. Likewise, the 3M dry medium culture plate (Petrifilm™ SM) has been evaluated as a method for determining population bacteria in raw milk and in fluid milk, respectively, by Gin et al. [4] and by McAllister et al. [5]. The comparison of the Petrifilm™ method for enumerating yeasts

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and molds (PYM) and for aerobic bacteria (PAC) to the conventional methods for enumerations of microorganisms in foods was studied by, among others, Beauchat et al. [6, 7] and by Chain and Fung [8] and White [9].

The purpose of this study was to compare the PetrifilmTM method for enumerating aerobic bacteria, coliforms, *Escherichia coli*, and yeasts + molds with conventional methods in different food products.

Materials and methods

Food products. Six homogeneous lots each of five food products (pasteurized milk, yoghurt ice cream, eggs, minced meat, fresh strawberries and frozen green beans) were analyzed.

Enumeration systems. Two systems were evaluated for enumerating mesophilic aerobic bacteria, coliforms, *E. coli* and yeast + mold population. The PetrifilmTM (Microbiology Products, 3M Health Care, St. Paul, MN 55144-1000) is a sample-ready system for the enumeration of microorganisms. The plate (7.5×9.5 cm) consists of a coating of agar medium on a base film which is overlaid with a second film coated with a cold-water-soluble gelling agent.

The other system utilized was the general enumeration procedures using plate count agar (PCA, Difco, Detroit, MI), violet red bile agar (VRBA, Oxoid, Basingstoke, Hampshire, England), eosin methylene blue agar (Levine EMB agar, Oxoid) and oxytetracycline-glucose-yeast extract agar (OGYE agar, Oxoid) to which appropriately diluted samples were applied using plating techniques.

Sampling procedure. Samples of 10 g or ml from each lot were analyzed in duplicate. Each sample was combined with 90 ml of 0.1 % Bacto peptone (Difco). Further dilutions were made as required. Samples were then serially diluted before depositing 1 ml aliquots in the centre of PetrifilmTM plates and in Petri dishes (90 cm in diameter).

Incubation and enumeration procedures. Inoculated PetrifilmTM plates and traditional agar media plates were incubated in an upright at 30 °C for 48–72 h, at 30 °C for 24 h, at 44 °C for 24 h, and at 25 °C for 5 d, for mesophilic aerobic bacteria, coliforms, *E. coli*, and yeasts + molds, respectively.

Statistical analysis. Kruskal-Wallis one-way analysis by ranks test were used to compare the enumeration methods in this study. Standard regressions techniques were used to calculate correlation coefficients, slopes, and intercepts (Stat graphics Statistical Graphics System. 1991. STSC, Inc. and Statistical Graphics Corporation).

Results and discussion

Mean counts of the different microorganisms detected in six food products using two enumeration systems are shown in Table I. The correlation coefficients, slopes and intercepts derived from comparing the two enumeration systems for each of the microbiological criteria are presented in Table II.

In five of the six foods tested (pasteurized milk, yoghurt ice cream, eggs, minced meat and fresh strawberries) the mesophilic aerobic bacteria counts on PetrifilmTM plates were higher than those obtained with PCA; only in the case of the frozen green beans did the opposite occur. In the four foods in which the presence of coliforms was detected (pasteurized milk, minced meat, fresh strawberries and frozen green beans), the counts made with the PetrifilmTM method also exceeded those on

Table I

Mean microbial counts detected in six food products using Petrifilm™ plates and conventional methods

Food products	Microbiological criteria	Mean counts* recovered (log ₁₀ CFU/g or ml)	
		Petrifilm™ plates	Conventional methods
Pasteurized milk (n=5)	Aerobic bacteria	2.69	2.30
	Coliforms	1.30	1
	<i>E. coli</i>	ND	ND
	Yeasts + molds	1.30	1.60
Yoghurt ice cream (n=5)	Aerobic bacteria	5.17	5.08
	Coliforms	ND	ND
	<i>E. coli</i>	ND	ND
	Yeasts + molds	ND	ND
Eggs (n=5)	Aerobic bacteria	2.53	ND
	Coliforms	ND	ND
	<i>E. coli</i>	ND	ND
	Yeasts + molds	1.77	2.30
Minced meat (n=5)	Aerobic bacteria	5.81	5.69
	Coliforms	5.60	5.58
	<i>E. coli</i>	ND	ND
	Yeasts + molds	5.47	5.48
Fresh strawberries (n=5)	Aerobic bacteria	2.71	2.64
	Coliforms	2.35	2.02
	<i>E. coli</i>	ND	ND
	Yeasts + molds	5.26	5.29
Frozen green beans (n=5)	Aerobic bacteria	5.34	5.49
	Coliforms	2.31	1.55
	<i>E. coli</i>	1	ND
	Yeasts + molds	2.82	3.86

* Comparisons of mean values of the enumeration methods for each microbiological criteria are made from a composite of six food product by Kruskal-Wallis one-way analysis by ranks test. In none of the cases were the differences significant ($P > 0.05$).

ND = Not detected

VRBA plates. *E. coli* was only observed in frozen green beans by the Petrifilm™ method and was not detected by the traditional method. Conversely, in the foods in which fungal contamination was verified i.e. all of them except the yoghurt ice cream, the enumeration of yeasts and molds on OGYE agar exceeded that obtained with the Petrifilm™ method (Table I).

By means of a Kruskal-Wallis one-way analysis by ranks test, the mean values obtained by the two enumeration procedures used (PetrifilmTM and the conventional one) were compared for the determination of each microbiological criterion (mesophilic aerobic bacteria, coliforms, and yeasts and molds) in the whole group of six foods. In no case were the differences significant ($P > 0.05$) (Table I).

Table II

*Statistical comparison in a composite of six food products using PetrifilmTM plates and conventional methods**

Measure of performance	Microbiological criteria	Petrifilm TM plates vs. conventional methods
Correlation coefficient	Aerobic bacteria	0.897
	Coliforms	0.861
	Yeasts + molds	0.981
Slope	Aerobic bacteria	0.599
	Coliforms	1.051
	Yeasts + molds	0.998
Intercept	Aerobic bacteria	4.673
	Coliforms	1.543
	Yeasts + molds	-0.813

* Comparison of mean values are made from a composite of six food products (pasteurized milk, yoghurt ice cream, eggs, minced meat, fresh strawberries and frozen green beans)

The correlation coefficients of PetrifilmTM aerobic bacteria, coliforms, and yeasts + molds versus PCA, VRBA and OGYE agar for each microorganism from a composite of six food products were, respectively, 0.897, 0.861 and 0.981; slopes were 0.599, 1.051 and 0.998; and intercepts were 4.673, 1.543 and -0.813 (Table II).

Chain and Fung [8] found a correlation of 0.999 between PetrifilmTM and aerobic plate count (APC) methods in chicken breasts, ground beef, ground pork and raw milk; while in pecans, thyme and whole wheat flour the correlation was 0.994. For these authors, the high correlation detected, higher than that obtained by us, may be attributed to the extreme care exercised in bacteriological manipulation in that research laboratory.

With regard to yeasts and molds, Beuchat et al. [6], in selected dairy and high-acid foods, obtained correlation coefficients which ranged between 0.992 and 0.995 between the PetrifilmTM and two traditional methods (APDA and CPCA). Likewise, Beuchat et al. [7] found, in 13 groups of food products (beans, corn meal, flour, fruit, a meat/vegetable entree, precooked meat, milk, nuts, pasta, potatoes, precooked sausage and a spice), correlations between PetrifilmTM plates versus APDA and CPCA plates

for recovering total yeasts and molds from a composite of the thirteen test foods, of 0.961 and 0.974, respectively. In both cases they were slightly higher or lower correlations than that found by us (Petrifilm™ YM versus OGYE agar: 0.981). Petrifilm™ YM plate counts were equivalent to or higher than conventional media for the enumeration of yeasts and molds in dried beans, flour, precooked beef and sausage, and lower compared to APDA and CPCA in the other nine test foods; no one method was best for all foods [7].

In our opinion, the results obtained with the use of the Petrifilm™ methods for the determination of mesophilic aerobic bacteria, coliforms, *E. coli*, and yeasts + molds in the groups of foods tested are satisfactory. Accordingly they maybe considered for use in routine food microbiological control.

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INTERACTION OF IMMUNOMODULANT SUBSTANCES IN MOUSE EXPERIMENTS WITH SPECIAL REGARD TO DRUG SENSITIVITY AND BACTERIAL TRANSLOCATION

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(Received October 11, 1994)

(Accepted November 18, 1994)

In acute toxicity experiments changes in drug sensitivity and in the rate of bacterial translocation (BT) were investigated in mice treated with immunomodulatory drugs: dianhydrogalactitol (DAG) in doses 20 and 30 mg/kg, chlorpromazine (CPZ) in doses 60 and 75 mg/kg and Mannozyim (M) in dose equivalent to 40 mg per kg zymosan. The drugs were used separately or in combination. The sensitivity of mice to immunosuppressive DAG or CPZ was higher in the case of combined treatment than that of separately treated ones. The rate of BT was also higher in mice receiving combined treatment.

Pretreatment with M exerting an immunostimulatory effect, influenced neither the sensitivity of mice to DAG or CPZ nor the very low normal rate of BT.

The present results reinforced the authors' earlier observations that the effects of immunosuppressive drugs cumulated in and caused more serious damage of the organism. The increase in drug sensitivity to immunosuppressive agents may be connected with an increased rate of BT and effect of endotoxin.

We demonstrated in earlier experiments [1, 2] that germ-free (Gf) mice were more sensitive to the lymphotropic drug dianhydrodulcitol (DAD) than conventional (Cv) mice of similar age. According to our further experiments, the calmodulin-antagonist chlorpromazine (CPZ) exerts a dose-dependent immunosuppressive effect and the sensitivity of Gf mice to CPZ is considerably inferior to that of Cv mice [3]. We attributed the increased sensitivity to (i) summation of immunosuppressive effects; (ii) bacterial translocation (BT), under the immunosuppressed condition into otherwise practically "sterile" inner organs and (iii) to endotoxin effect.

In earlier experiments we found in Cv mice an increased drug sensitivity (DS) caused by microorganisms of immunosuppressive capacity and their metabolites, such

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as LCM virus [6], a bacterial endotoxin preparation [7] and *Bordetella pertussis* vaccine [8]. Conditions elicited by artificial interventions including GvH reaction [9], wasting syndrome following neonatal thymectomy [10] or condition subsequent to stress effect [11] were also followed by an increased DS in Cv mice. We have shown, in accordance with other authors, that Mannozy (M), a glucomannan prepared from *Saccharomyces cerevisiae*, elicited a significant splenic hypertrophy and, in addition, proved to be an immunostimulant [12].

In the present experiment, we made attempts to show whether

- in mice kept conventionally, combined treatment with immunosuppressive agents, such as dianhydrogalactitol (DAG) - a stereoisomer of DAD - and CPZ elicits a change in sensitivity to these agents;
- there is a change after separate use of both of these drugs in the degree of BT;
- M, an immunostimulant, exerts an effect influencing DS, and whether
- BT can be demonstrated in splenic hypertrophy caused by M.

Materials and methods

Experimental animals. The Cv mice were CD-1 (Charles River Co.) mice of both sexes 6 to 8 weeks of age.

DAG treatment. DAG, the epoxide of DAD (Chinoïn, Budapest) is a cytotoxic drug belonging to the alkylating agents. The substances were dissolved in sterile distilled water and used for the experiment in half an hour. The degree of dilution was 0.01 ml/g body weight. The mice were inoculated intraperitoneally (i.p.), each on one occasion.

CPZ treatment. A 2.5% aqueous solution of chlorpromazinum hydrochloricum (EGIS, Budapest) was used. Further dilutions, necessary for the different doses, were prepared in PBS immediately before use. The mice were inoculated i.p. on one occasion each.

Mannozy treatment. The preparation Mannozy (Institute for Vaccine Production and Research HUMAN, Budapest) contains 1 mg of the polysaccharide of *S. cerevisiae* suspended in 1 ml of saline (0.1% suspension of zymosan). It contains no preservative [13, 14]. The active agent is a water-insoluble glucomannan polysaccharide of *S. cerevisiae*, built up of glucose and, mainly, of mannose molecules. The mice were inoculated i.p., each on one occasion. The dose is equivalent to 40 mg/kg of zymosan calculated for mice of 25 g body weight.

Control groups. These received PBS in a volume corresponding to the volume of DAG, CPZ and M.

Examination of bacterial translocation. Preparation of cell suspension. After the mice were killed, two mesenteric lymph nodes, the spleen and the liver were removed with sterile instruments. Cell suspension was prepared in 0.5 ml PBS from each of the lymph nodes and from a 100 mg fragment of each spleen and liver.

Isolation of bacteria. The whole quantity of each of the cell suspensions was inoculated each into a serum broth medium. Subcultures were prepared on blood agar at 24 h intervals. The colonies growing under aerobic conditions were submitted to macroscopic, microscopic and biochemical examination. The instructions given by the "ATB Expression Antibiotic Sensitivity Determination System" were followed in the identification of bacteria. The isolation experiments were repeated on every fifth day. An experiment was considered negative when no bacterial growth was observed on the blood agar media 48 h after inoculation.

Determination of translocation index:

$$\text{Translocation index} = \frac{\text{translocation in the treated group \%}}{\text{translocation in the control group \%}}$$

Calculation of relative spleen weight:

$$\text{Relative spleen weight} = \frac{\text{spleen weight mg}}{\text{body weight g}}$$

Calculation of spleen index:

$$\text{Spleen index} = \frac{\text{average rel. spleen weight for the treated group}}{\text{average rel. spleen weight for the control group}}$$

Statistical evaluation. Student's *t*-test was applied. Limit of significance: $P=0.05$.

Experiments and results

Three different series of experiments (I, II and III) were carried out.

In Experiment I we measured the DS and the BT index after DAG+CPZ treatment. Two groups of mice were injected with two different doses separately and in combination on concomitant days (experimental groups I/A, I/B and I/C). The data for the groups and the number and treatment of mice are shown in Table I.

Table I

Treatment of mice in Experiment I

Groups of mice	No. of mice	Treatments i.p.		PBS
		DAG (mg/kg)	CPZ (mg/kg)	
I/A DAG	20	30	—	+
CPZ	20	—	50	
DAG+CPZ	20	30	50	
C	20	—	—	
I/B DAG	20	20	—	+
CPZ	20	—	60	
DAG+CPZ	20	20	60	
C	20	—	—	
I/C DAG	14	30	—	+
CPZ	10	—	50	
DAG+CPZ	14	30	50	
C	4	—	—	

In groups I/A and I/B, the observation time was 21 days. The number of deaths was registered for each group. The results expressed in the time and per cent of mortality are shown in Figs 1 and 2.

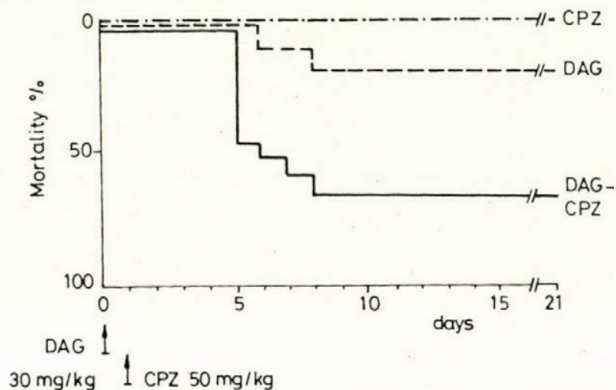


Fig. 1. Mortality curves for mice pretreated with CPZ and/or DAG in Experiment I/A

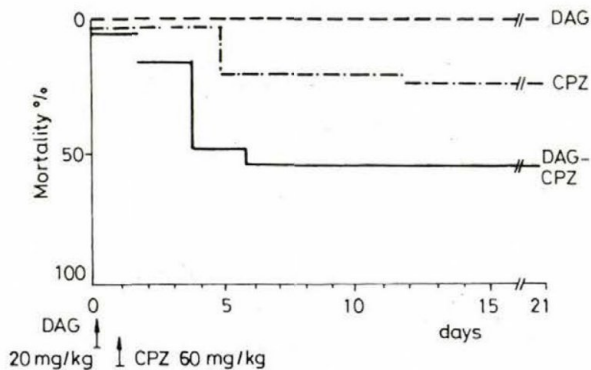


Fig. 2. Mortality curves for mice pretreated with DAG and/or CPZ in Experiment I/B

In Fig. 1 it is clearly seen that 50 mg/kg CPZ, a dosage causing no deaths when given alone, and 30 mg/kg DAG, which caused 20% mortality when administered separately, but 65% mortality when the two drugs were given in combination. Figure 2,

on the other hand, shows that 20 mg/kg DAG causing no mortality and 60 mg/kg CPZ causing 21% mortality when given separately, induced 50% mortality when given in combination. Accordingly, the drugs given in combination elicited an elevated mortality, compared to their separate administration. There was no mortality in group C during the observation period.

In group C experiment I, we followed up the BT. By the 6th day following the DAG treatment 50% of the mice in the DAG+CPZ group had died, whereas there was no mortality in the other groups at all. The surviving mice were killed on day 6, and isolation of bacteria was attempted from two peritoneal lymph nodes, spleen and liver of each mouse. The results are presented in Table II.

Table II

Bacterial translocation in Experiment I/C

Group of mice	Translocation				Translocation index	
	frequency in mice*	%	frequency in organs**	%	mice	organs
DAG	12/14	85	28/42	66.6	3.4	8.2
CPZ	5/10	50	3/30	26.6	2.0	3.2
DAG+CPZ	7/7	100	17/21	81.0	4.0	10.1
C	1/4	25	1/12	8.3	—	—

* No. of positive mice / no. of examined mice

** No. of positive mice / no. of examined organs

BT occurred the most frequently in the group of mice given the combined treatment; it amounted to 85% when calculated for organs and to 100% when calculated for mice. Similarly to the data of frequency, the translocation index was the highest for groups of mice given the combined treatment.

Sixty-one bacterial strains were isolated altogether. Of these, 27 were Gram-negative (*Escherichia coli*, *Proteus mirabilis*), the remaining 34 strains were Gram-positive (*Staphylococcus*, *Micrococcus* and *Streptococcus* spp.). There was no significant difference in the distribution in the different groups.

In Experiment II the effect of the immunostimulant agent M on the sensitivity to DAG and CPZ was examined. Mice pretreated with M were treated with DAG and/or CPZ on the subsequent day. The mice in group C were given PBS instead of drug. Twenty animals were assigned to each group. The results are presented in Table III.

Table III

Treatment of mice in Experiment II

Group of mice	Treatments i.p.			
	Zymosan mg/kg	CPZ mg/kg	DAG mg/kg	PBS
M	40	—	—	
CPZ	—	75	—	
M+CPZ	40	75	—	
DAG	—	—	40	
M+DAG	40	—	40	
C	—	—	—	+

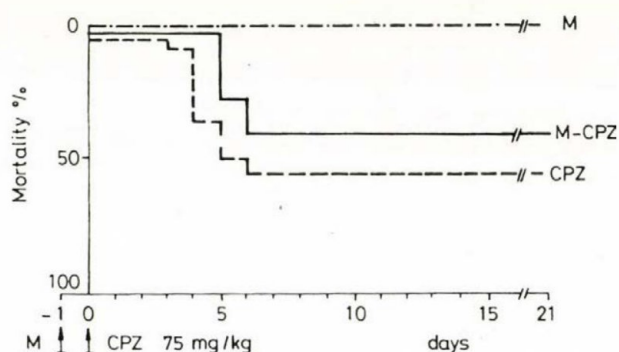


Fig. 3. Curves illustrating mortality rate in Experiment III

During the 21-day observation period, mortality was registered daily (Figs 3 and 4).

The sensitivity, either to DAG or to CPZ, was not influenced by the M pretreatment; neither the mortality rate nor its progress in time was influenced.

During the observation period there was no mortality in either of groups C and M. Ten mice in group M were sacrificed on the 7th day following pretreatment. For the killed mice, the relative spleen weight and the spleen index were determined. The results in Table IV show that a significant splenic hypertrophy developed in the mice pretreated with M.

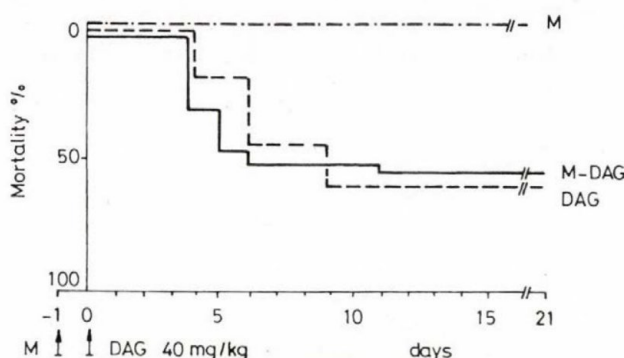


Fig. 4. Curves illustrating mortality rates in Experiment III

Table IV

Splenic hypertrophy caused by M

Group of mice	Relative spleen weight*	Spleen index
M	8.75±1.63	1.8
C	4.85±1.02	1.0

* Mean±S.D.

M versus C: $p < 0.001$

In Experiment III, the effect of M pretreatment on BT was examined. Ten mice from group M and 10 from group C were killed on the 7th day following M treatment in order to use them in isolation experiments.

Table V shows the degree of positivity for the examined organs, i.e. the frequency of BT in the different groups. On the effect of M pretreatment neither the percentage values nor the translocation indices showed any change. These results are in accordance with mortality data of experiment II. In other words, M caused no change in the mortality induced by DAG or CPZ, i.e. did not change the sensitivity to these drugs and, corresponding to these findings, it did not influence the BT rate found in the groups of mice given no such pretreatment.

Table V

Bacterial translocation in Experiment III

Group of mice	Translocation				Translocation index	
	frequency in mice*	%	frequency in organs**	%	mice	organs
M	2/10	20	4/30	13	1.0	1.3
C	2/10	20	3/30	10	1.0	1.0

* No. of positive mice / no. of examined mice

** No. of positive mice / no. of examined organs

Discussion

In a part of the present work, we performed acute toxicity experiments in which the mortality rates for groups of mice pretreated with different agents were comparatively measured. In this way we were capable of drawing conclusion on the changes occurring in drug sensitivity following various interventions. In further parts of our work, we utilized Berg's BT phenomenon [15] in order to explain our results obtained in the acute toxicity experiments.

In Experiment I, it was shown that the mortality rate was by 30% (Experiment I/A) and by 55% (I/B) higher after combined treatment with DAG and CPZ than after simple DAG treatment. As compared to the mortality rates following simple CPZ treatment, the combined DAG+CPZ treatment was followed by a mortality increased by 65% (I/A) or 34% (I/B). We attempted to explain these considerable differences by the BT phenomenon and by endotoxin effect, for after DAG+CPZ treatment we had shown translocation of bacteria from the intestinal flora, in a dose-dependent quantity, into the otherwise practically "sterile" inner organs; bacteria were isolated from the peritoneal lymph nodes, from the spleens and from liver samples [3, 4].

The present isolation experiments support our concept on the role of translocation, suggesting that in mice having been submitted to double pretreatment, BT-positive organs were of increased number. We have assumed on the basis of our earlier results [7] that in the BT-positive groups the endotoxin effect was also elevated, and the endotoxin contributed to the increased mortality rate, i.e. to the increased DS.

According to Experiment II, pretreatment with M, i.e. with an immunomodulant agent, induced no increase in the sensitivity to either of DAG and CPZ, and the splenic hypertrophy elicited by the M treatment influenced neither the DS nor the BT, the latter being phenomenon that exists in normal Cv mice but rarely. An alternative interpretation of our results may be that the DS did not change because the M pretreatment failed to elicit BT.

The present experiments agree well with our earlier experiences: the harmful side-effects of different interventions causing immunosuppression may summarize and may lead to an unexpected injury in higher organisms as well.

Acknowledgement. This work is supported by Hungarian Research Fund (OTKA I/3.2612 "A").

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THE NUMBER OF GLUCOCORTICOID RECEPTORS IN PERIPHERAL HUMAN LYMPHOCYTES IS ELEVATED BY A ZINC CONTAINING TRACE ELEMENT PREPARATION

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(Received October 18, 1994)

(Accepted November 23, 1994)

A trace element preparation (Béres Drops Plus®, BDP) elevates the number of glucocorticoid receptors (gcR) in peripheral lymphocytes isolated both from healthy blood donors and rheumatoid arthritis patients. This enhancement by BDP was found either for constitutive expression of gcRs or in experiments when the lymphocytes were stimulated by interleukin (IL)-6. There was no significant effect of BDP on IL-1 and tumour necrosis factor alpha (TNF alpha)-induced changes of gcRs. The effect of BDP was greatly dependent on the presence of Zn^{++} ions in the preparation, since the augmenting effect was abolished if BDP did not contain zinc.

The bilateral interaction between the interleukin-6 (IL-6) and functionally related other inflammatory cytokines as well as the hypothalamo-pituitary-adrenal axis represents a separate facet of complex web of regulatory neuroendocrino-immunological interactions. The activation of hypothalamo-pituitary-adrenal axis results in increased production of glucocorticoids with anti-inflammatory activity. Glucocorticosteroids are among the most effective drugs used in the therapy of the inflammatory (e.g. rheumatic) diseases [1]. They bind to intracellular receptors resulting in a translocation to the nuclei with the subsequent modification of gene expression pattern of the cell. The antiinflammatory effects of glucocorticoids involve numerous pathways, including a strong negative effect on inflammatory cytokines IL-1 beta, IL-6 and TNF alpha [2] as well as up-regulation of cytokine receptors [3].

On the other hand IL-1 beta, IL-6 and TNF alpha elevate serum adrenocorticotrop hormone (ACTH) level indirectly through the stimulation of the corticotropin-releasing hormone (CRH)-secreting neurones in the hypothalamus and directly as intrapituitary releasing factors [4]. Moreover, a definite enhancement of the

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number of glucocorticoid binding sites (i.e. glucocorticoid receptors-gcR) by these inflammatory cytokines has been proved recently [5].

Our present data provide evidence that a trace element preparation (Béres Drops Plus®, BDP) acts similarly to cytokines mentioned above, since it is able to elevate the number of gcRs. Moreover BDP further potentiates the stimulatory effect of IL-6, but has no significant effect on the effectiveness of IL-1 beta and TNF alpha. We found that the presence of zinc in BDP is substantial for this effect.

Materials and methods

Reagents. For the experiments a trace element mixture (Béres Drops Plus®, BDP) was used. The composition of this preparation is given in Table I. The mixture was provided by the Béres Co. Recombinant human IL-6, IL-1 and TNF alpha preparations were purchased from Sigma Chemical Co. St. Louis, MO.

Cells and incubations. Peripheral lymphocytes from Na-EDTA treated venous blood of healthy volunteers (n=9) and rheumatoid arthritis (RA) patients (n=7) were isolated on Ficoll-Hypaque gradient and plastic adherence by conventional laboratory protocol. RA patients were all in active onset of disease (strongly positives in gamma-latex and Waaler-Rose tests).

A total of 2×10^6 cells were incubated in a culture medium (RPMI-1640, 10% fetal calf serum, 2 mM glutamine, 2 g/l NaHCO_3 , 5000 U/l penicillin G sodium, 5 mg/l streptomycin (Gibco, Paisley, UK) for 24 h at 37 °C in the presence of BDP (1:25 dilution, based on preliminary experiments) with or without optimal concentrations of human recombinant IL-1 beta (10 U/ml), IL-6 (50 U/ml) and/or TNF alpha (25 U/ml).

Assay of glucocorticoid binding. Glucocorticosteroid binding was determined by the method of "whole cell uptake" [6]. Cells were washed 3 times with SSC (0.15 M NaCl, 0.015 M Na-citrate pH 7.4) at 0 °C, then duplicate samples of 2×10^6 cells were incubated in medium RPMI-1640 in the presence of 7.2×10^{-8} M (1,2,4,(n)-3H) triamcinolone acetonide (Amenham, Buckinghamshire, UK.), without (for determination of total binding) or with (for determination of unspecific binding) a 500-fold molar excess of unlabelled triamcinolone acetonide (Sigma) at 37 °C for 40 min. Washed cells were solubilized in toluene based scintillation cocktail containing 30% Triton X-100. Specific binding was calculated as the difference between total and unspecific binding. The counts were normalized to 1000 cells, and the results were expressed as percentage of the value of the untreated cells.

Statistical analysis. Values shown are means and standard error of the means. Student's t-test was used in calculating p values. In the legend for Fig. 1 □ and □□ represent p values for differences between cytokine treated or untreated cells, * and ** mean p values for differences between BDP treated or untreated cells.

Results

Figure 1 shows the effect of BDP on the number of glucocorticoid binding sites (gcR) in peripheral lymphocytes of healthy (Fig. 1A) and RA patients (Fig. 1B), respectively. In healthy individuals both IL-6, IL-1 beta and TNF alpha significantly increases the number of gcRs (□ = $p < 0.05$, □□ = $p < 0.01$). When the effect of BDP is studied, it is clear, that even BDP alone elevates the number of gcRs (control). Moreover, it further increases the stimulatory effect of IL-6 (* = $p < 0.05$, ** = $p < 0.01$).

However, though it acts in the same tendency, there is no significant effect on the stimulatory action of IL-1 beta and TNF alpha.

To check the significance of Zn in BDP, we obtained Zn free trace element preparation. Results in Table II show that the presence of Zn is critical in BDP.

Table I

The composition of Béres Drops Plus® (BDP) preparation

Element	mmol/l
Fe	36
Zn	17.4
Mn	5.56
Cu	4.0
Mo	1.96
V	2.39
Ni	1.85
B	9.7
F	4.76
Co	0.42

Table II

The significance of Zn in the effect of BDP on glucocorticosteroid binding

	Exp. 1	Exp. 2	Exp. 3
Control	4.1	5.1	3.9
IL-6 (50 U/ml)	7.4	8.1	7.6
BDP (1:25)	6.0	6.9	6.1
BDP without Zn (1:25)	4.0	5.2	3.8
IL-6 + BDP	8.9	9.4	9.0
IL-6 + BDP without Zn	7.5	8.3	7.6

(cpm $\times 10^{-3}$)/ 10^6 cells; ^3H -triamcinolone-acetonide binding (for details see: Materials and methods)

BDP: Béres Drops Plus®

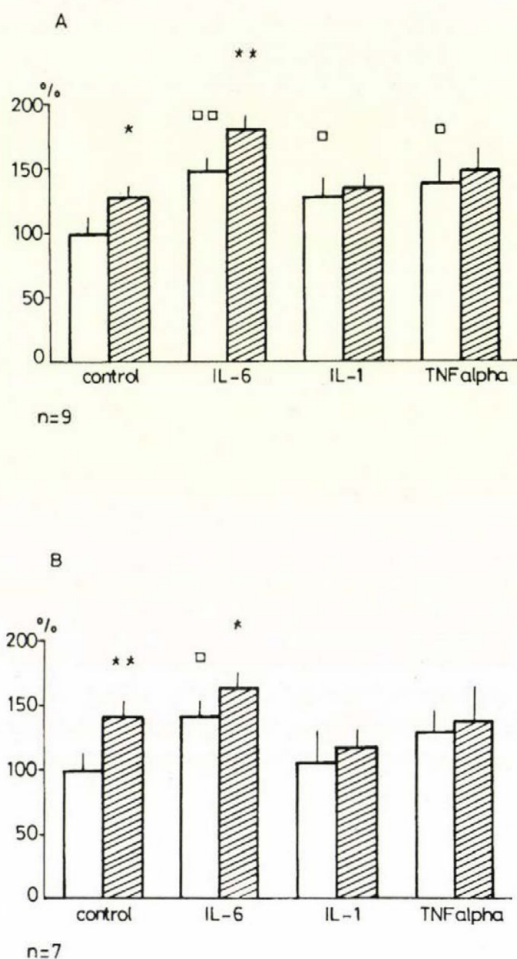


Fig. 1. The effect of Béres Drops Plus® (BDP) on the number of glucocorticosteroid receptors in human peripheral lymphocytes of healthy volunteers (A) and rheumatoid arthritis (RA) patients (B). The cells were treated with BDP (in a dilution of 1:25) or with 10 U/ml IL-1 beta, 50 U/ml of IL-6 and 25 U/ml TNF alpha at 37 °C for 24 h. For details of incubation and glucocorticoid binding assay see Materials and methods. The statistical analysis for cytokine effects are shown by □, that of BDP effects are represented by * (□ = $p < 0.05$, □□ = $p < 0.01$; * = $p < 0.05$, ** = $p < 0.01$). Open columns, medium; shaded columns, BDP

Discussion

The biological significance of trace elements in the immune response is abundantly studied. It is known, that the deficiency of Cu^{++} ions, significantly impairs the activation of B lymphocytes [7] while some ions (e.g. Zn^{++}) are stimulatory for T cell proliferation [8]. Cytokine production is also affected by ions, e.g. the Fe^{++} ions play basic role in the IL-1 BETA production [9] and Zn, Se and Cu are very important in the action of thymic hormones [7].

Previous observations suggest a definite functional effectiveness of the Béres Drops Plus® (BDP) preparation in various immunodeficiencies. In this study the effect of this mixture of trace elements with a well-characterized composition was studied on the expression of glucocorticoid receptors. The actual number of gcRs provides the "glucocorticoid sensitivity" of these cells. Since this parameter of cells determines their effectiveness for glucocorticoid hormones, every factors, which influences this feature is highly important for the regulation of the cell.

Our studies show, that the positive effect of BDP on the expression of gcRs resembles to that of IL-6, IL-1 beta and TNF alpha. This effect is rather considerable – it is close to 25% in healthy and 40% in the lymphocytes of RA patients. Moreover, it can further enhance the effect of IL-6 (with an approx. 20% or 15 % in healthy donors and RA patients, respectively). Our experiments proved, that the presence of Zn is critical for this effect.

These data reveal, that trace elements, like Zn are very important in fine tuning of immune and inflammatory response, just by modification of glucocorticoid sensitivity of target cells. Further studies are required to elucidate the molecular mechanism of the action of Zn and other trace elements on the biosynthesis of gcRs and on steroid metabolism, generally.

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INTERACTION BETWEEN TRICYCLIC PSYCHOPHARMACONS AND SOME ANTIBIOTICS*

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(Received November 3, 1994)

(Accepted November 30, 1994)

The tricyclic psychopharmacons, e.g. clozapine, promethazine and imipramine cure plasmids and inhibit plasmid transfer among bacteria due to the inhibition of supercoiling activity on DNA gyrase. In addition an interaction was found between clozapine, imipramine, promethazine and some antibiotics, e.g. penicillins and tetracycline in vitro. The nature of interaction is based on a charge transfer complex, which is formed between clozapine, imipramine, promethazine and penicillins. Differential spectrophotometry showed that ampicillin reduced the highest energy peaks of clozapine, promethazine and imipramine. Streptomycin did not alter the spectrum of clozapine; however, tetracycline somewhat reduced all the peaks of clozapine. Clozapine and promethazine exhibited a synergistic effect with ampicillin, tetracycline and gentamicin on *Escherichia coli* cells in in vitro. This kind of interaction was missing in the case of imipramine.

For many years spot observations have shown that severe pyogenic infections in psychiatric patients responded well to antibiotic treatment. All of these patients, in combination with antibiotics received a heavy dosage of chlorpromazine. No side effect of the combination was noted and no contraindication was mentioned [1]. A similar beneficial interaction was found between promethazine and gentamicin [2–7]. The clinical importance of this interaction between phenothiazines and antibiotics was

*This paper is dedicated to Professor Ilona Béládi, director emeritus of the Institute of Microbiology of Albert Szent-Györgyi University Medical School in Szeged, Hungary, by her pupils on the occasion of her 70th anniversary.

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discussed by Kristiansen [8], Kásler et al. [2, 3] and Molnár et al. [6]. Surprisingly, it was observed that promethazine and other phenothiazines were able to eliminate the antibiotic resistance of bacteria in vitro [4, 9] and in vivo [2, 3, 6].

After sporadic observations a systematic study was initiated on the antibacterial effect of phenothiazines and related compounds in vitro [5, 9, 10]. Beside direct antibacterial action, the drugs act on bacterial pili [11] which may play a role in the pathogenicity of bacteria. The adhesion of *Escherichia coli* strains to tissue culture cells was inhibited and the ultrastructure of bacteria also changed in the presence of the phenothiazines and related compounds [12, 13].

The morphological changes resembled the antibacterial effect of penicillin [10]. Beside the phenothiazines other drugs, e.g. dibenzo-azepines and dibenzocycloheptanes were found to have antibacterial effects in vitro.

Clozapine, imipramine and promethazine are being used in psychiatry all over the world. There are numerous publications on the various side effects in vivo, however, paper of Csík [14] except the recent, none of the published side effects are associated with the drug interaction with antibiotics. Therefore we decided to analyse the interaction of clozapine, imipramine, promethazine and some representative antibiotics in vitro. In this paper we report the interaction between clozapine, imipramine, promethazine and some selected antibiotics.

Materials and methods

Compounds. Clozapine (Leponex) was from Sandoz, promethazine (Pipolphen), imipramine (Melipramine) were from EGIS, Budapest, Hungary.

Antibiotics. Ampicillin (Penbritin, Beecham, UK), Gentamycin (Biogal, Debrecen, Hungary), Streptomycin (EGIS, Budapest, Hungary), Tetracyclin (Chinoïn, Budapest, Hungary), and Penicillin-G (Biogal, Debrecen, Hungary) were used.

Bacterial strains. *E. coli* K12 LE 140 F⁺lac and *E. coli* K12/R144 Km^r drd-3 were provided by I. B. Holland (Department of Genetics, University of Leicester, UK), *E. coli* K12 W1 azi^r plasmidless derivative carrying sodium azide resistance on the chromosome (provided by Judith Lantos, Csongrád Country Institute, National Public Health Service, Szeged) was used as a recipient in transconjugation experiments.

Media. For culturing the bacteria tryptone yeast extract (MTY) broth and agar plates were prepared according to Alföldi et al. [15]. Eosin methylene blue (EMB) agar was used for detection of plasmidless (lactose-negative) bacteria in the *E. coli* F⁺lac (lactose-positive) population.

Elimination of the F⁺lac plasmid. Plasmid elimination was carried out as described by Molnár et al. [4] and Mándi et al. [16]. An overnight preculture of *E. coli* K12 F⁺lac was diluted 10⁻⁴ fold and 0.05 ml (about 5 × 10³) bacteria was used to inoculate 5 ml MTY broth. Different concentrations of the tested drugs were added and the tubes were incubated at 37 °C for 24 h. From the tubes showing growth, dilutions were prepared and 0.1 ml samples were plated on EMB or MTY agar. The plates were incubated at 37 °C for 24 h then counted for lac⁻ (plasmidless) and lac⁺ (plasmid carrying) bacterial colonies.

Inhibition of R-plasmid transfer. *E. coli* K12/R144 was used as donor and *E. coli* K12 Na-azide resistant as recipient. From overnight precultures of the two strains grown without aeration at 37 °C 100-fold dilutions were prepared in MTY media and incubated at 37 °C for 4–6 h. When absorbance at 620 nm was 0.4, donor and recipient cell cultures were each diluted 10-fold in MTY media and equal volumes were mixed and incubated without aeration. After 5 min the drugs were added to the mating pairs at concentration of 0.5 MIC. The samples were incubated at 37 °C for 24 h then vigorously

shaken and diluted. Further dilutions were made and 0.1 ml aliquots were plated onto selective agar plates. MTY agar containing 50 µg/ml kanamycin plus 400 µg/ml sodium azide was used for the estimation of Km^r plus Na-azi^r resistant transconjugants. The number of donors was determined on kanamycin and the recipients on sodium azide containing MTY agar. The plates were incubated at 37 °C for 48 h and the colonies were counted [9, 11].

DNA gyrase activity. The reaction mixture [17] containing relaxed pBR 322 plasmid DNA (100 ng), 1 U of *Micrococcus luteus* DNA gyrase (Bethesda Research Laboratories) and drug solution was incubated at 37 °C for 1 h. The reaction was stopped at 0 °C by the addition of 1% (v/v) SDS. Samples were loaded onto a 0.8% agarose gel and run at 60 V for 3 h. Gels were stained with ethidium bromide and photographed under UV light (254 nm).

Differential spectrophotometric investigation of clozapine, imipramine, promethazine and antibiotics. The complex formation between antibiotics and clozapine was investigated as follows. Antibiotic solutions were distributed at 450 µl into Eppendorf tubes and the experimental drugs were added to the antibiotics in 50 µl. Aliquots of 450 µl Tris-HCl (0.015 M pH 7.0) buffer plus 50 µl drug solutions were used as blanks in differential spectrophotometry. The spectral changes of clozapine, imipramine and promethazine in the presence of the antibiotics were measured from 200 to 450 nm wavelength on an SP 800 Unicam spectrophotometer [18].

Interaction between some psychopharmacons and antibiotics in the antibacterial effect. Filter paper strips were impregnated with clozapine, imipramine and promethazine at a concentration of 5000 µg/ml distilled water. Ampicillin, gentamicin and tetracycline were used in 1000 µg/ml; 1×5 cm filter paper strips were immersed into the solutions and after removal they were allowed to dry at room temperature.

E. coli LE 140 overnight broth culture was plated on the surface of Müller-Hinton agar and one psychopharmaccon and one antibiotic strip were placed on the inoculated agar surface at right-angles to one another. The plates were then incubated at 37 °C for 16 h, then the shape of the inhibition zones was evaluated.

Results

Previously it was found that ampicillin enhanced or modified the clinical effect of clozapine and for this reason the interaction between similar drugs was investigated in vitro as well. The spectrum of clozapine changed in the presence of ampicillin. Ampicillin affects the high energy peaks of clozapine at 215 and 236 nm [14]. The other two antibiotics, as representatives of their classes, streptomycin and tetracycline were also tested by differential spectrophotometry. Streptomycin was not able to change the spectrum of clozapine, however, tetracycline reduced all the peaks [14]. In further spectrophotometric studies it was found that ampicillin reduced the highest energy bands of promethazine (Fig. 1) and imipramine (Fig. 2). The corresponding energy values were calculated by Einstein's equation, as follows:

$$E = \frac{h \times c \times N}{\text{nm}} = \frac{286000}{\text{nm}}$$

(h=Planck constant, c=velocity of light, N=Avogadro number)

After the differential spectrophotometric study, the interaction of clozapine and two other tricyclic drugs with antibiotics were studied on bacteria. The question was,

whether tricyclic psychopharmacoons and some representative antibiotics reduce or enhance the antibacterial effects of the antibiotics. The psychopharmacoons were applied at a concentration below the antibacterial effect and then the system was prepared to estimate the synergy or antagonism between the psychopharmacoons and antibiotics.

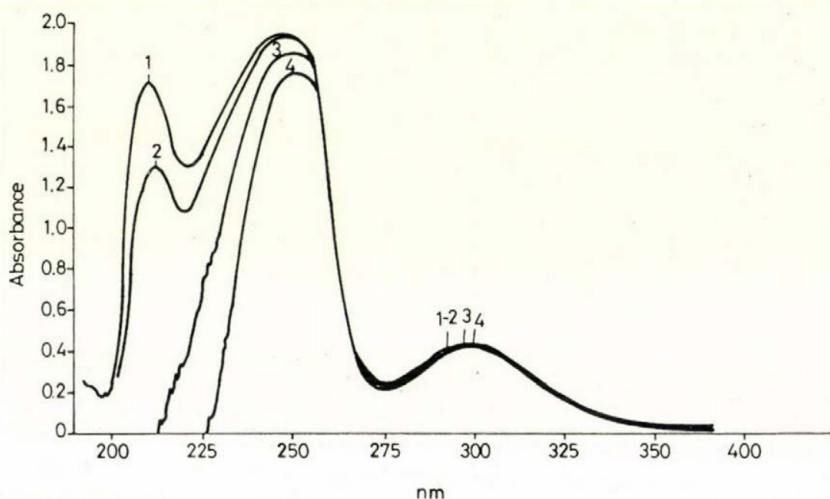


Fig. 1. The effect of ampicillin on the spectrum of promethazine. 1. Promethazine 50 µg/ml + water, blank: water; 2. Promethazine 50 µg/ml + ampicillin 5 µg/ml, blank: ampicillin 5 µg/ml; 3. Promethazine 50 µg/ml + ampicillin 50 µg/ml, blank: ampicillin 50 µg/ml; 4. Promethazine 50 µg/ml + ampicillin 100 µg/ml, blank: ampicillin 100 µg/ml

We found synergy between clozapine and ampicillin, promethazine and ampicillin, however, we failed to find synergy between imipramine, ampicillin and tetracycline. The synergy between imipramine and gentamicin is weak. The antibacterial effect of gentamicin was also enhanced by clozapine and promethazine. The antibacterial effect of tetracycline was not enhanced by imipramine and promethazine, although clozapine exerted a weak synergistic effect (Table I) in the agar diffusion test. Clozapine and promethazine could enhance the permeability of bacterial membrane to the tested antibiotics.

Nevertheless, clozapine affected the genetical base of antibiotic resistance by inhibiting bacterial plasmid replication in model experiments (Table II). The antiplasmid effect of clozapine was concentration dependent. In addition to the inhibition of plasmid replication in bacteria, clozapine also inhibited the transfer of antibiotic resistance plasmid from one bacterial strain to another (Table III). The number of donor and recipient cells decreased to a lesser extent than the frequency of newly formed plasmid containing cells, which means that there was a selective

inhibition of plasmid transfer in the presence of clozapine. The inhibitory action on preformed mating pairs of bacteria showed that transconjugal DNA synthesis was probably inhibited by clozapine.

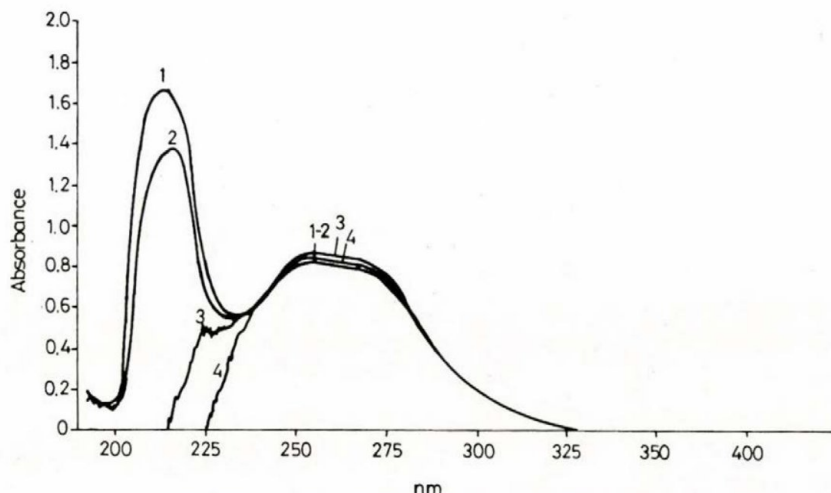


Fig. 2. The effect of ampicillin on the spectrum of imipramine. 1. Imipramine 50 $\mu\text{g/ml}$ + water, blank: water; 2. Imipramine 50 $\mu\text{g/ml}$ + ampicillin 5 $\mu\text{g/ml}$, blank: ampicillin 5 $\mu\text{g/ml}$; 3. Imipramine 50 $\mu\text{g/ml}$ + ampicillin 50 $\mu\text{g/ml}$, blank: ampicillin 50 $\mu\text{g/ml}$; 4. Imipramine 50 $\mu\text{g/ml}$ + ampicillin 100 $\mu\text{g/ml}$, blank: ampicillin 100 $\mu\text{g/ml}$

One of the key enzymes in plasmid replication – the DNA gyrase that introduces the negative superhelical turns into plasmid DNA – was also affected. Clozapine, imipramine and promethazine inhibited the gyrase (Table IV), showing that the drug disturbs the establishment of the tertiary structure of extrachromosomal genetic elements in bacteria.

Discussion

It is possible that clozapine, imipramine and promethazine have multifocal action in bacterial infections. Beside the direct anti-bacterial action which was proved in experiments *in vitro* the drug was able to eliminate the antibiotic resistance of multiple resistant bacterial cells both *in vitro* and *in vivo* [7]. The drug has some synergistic effect with several antibiotics and, in addition, can enhance the entrance and permeation of antibiotics into the infected cells throughout the cell membrane [19,

13]. A possible explanation of our experience with the tricyclic drugs plus antibiotics in the treatment of chronic bacterial infections could be the synergistic effect of the drug with the antibiotics. In a very few cases the elimination resistance of bacteria was also observed [7].

Table I

Interaction between psychopharmacoens and antibiotics in the antibacterial effect in agar diffusion technique on E. coli

Psychopharmacoens	Antibiotics	Type of interaction	Notes
Imipramine +	None		
	Ampicillin	Indifferent	
	Gentamicin	Weak synergy	
	Tetracycline	Indifferent	
Clozapine +	None		Haemolysis
	Ampicillin	Synergy	Haemolysis
	Gentamicin	Synergy	Haemolysis
	Tetracycline	Weak synergy	Haemolysis
Promethazine +	None		
	Ampicillin	Synergy	
	Gentamicin	Synergy	
	Tetracycline	Indifferent	

Table II

Effect of clozapine on the stability of Flac plasmid in E. coli

Clozapine µg/ml	Plasmid elimination %	No. of cells ($\times 10^7$) after 24 h incubation
0	0	5.6 – 28.0
10	0.05 – 0.6	4.9 – 11.8
20	0.40 – 1.2	4.8 – 9.6
30	0.50 – 1.8	4.5 – 9.2
40	2.90 – 3.6	3.6 – 6.5
50	4.70 – 6.2	5.1 – 5.4
60	1.20 – 5.0	2.5 – 7.5
80	0.20 – 1.6	1.1 – 0.63
100	MIC	–

Table III

Effect of clozapine on Km^r plasmid transfer to az^r recipients E. coli

Concentration µg/ml	No. of recombinants transconjugants × 10 ³	No. of donor Km ^r cells × 10 ⁵	No. of recipient Na-azide cells × 10 ⁵
0	2.03	8400	190
10	0.85	8150	180
25	0.71	7000	160
40	0.42	3500	105
50	0.11	1250	84

Table IV

The dose dependent inhibition of DNA gyrase of M. luteus by clozapine, imipramine and promethazine

Compounds concentration µg/ml		Enzyme activity
Clozapine	1000	0
	500	0
	100	+
Imipramine	1000	0
	500	0
	100	+
Promethazine	1000	0
	500	0
	100	+
Control (without drug)		+

In a previous study the antibiotic sensitivity of bacterial strains isolated from patients who regularly took tricyclic psychopharmacons was compared to patients who were not treated with psychopharmacons. From the first group of 50, 12 patients had significant bacteriuria, whereas from the control group 21 of the 50 patients had significant bacteriuria [7].

When antibiotic sensitivities of bacteria isolated from drug treated and untreated patients were compared, it was found that the resistance to nalidixic acid (75%) and

oxolinic acid (66%) was higher in the drug treated group than in the group not treated with psychopharmacoans (25% and 33% [7]).

When the antiplasmid effect of clozapine was examined in patients who regularly took these medicines, it was found that the resistance patterns of 10 bacterial isolates in the urine were different.

The first studies of successful plasmid elimination by promethazine in human patients were published in 1982 [3] and recently [6, 7]. This type of interaction may explain the beneficial interaction in bacterial infections. However, the apparently dangerous in vivo interaction of clozapine and penicillin cannot be explained as simply as that [14]. Naturally, there are some other possibilities to explain the drug-antibiotic interactions, e.g. on the receptor level one of the compounds can modify the binding of the other.

Apart from plasmid curing there is another way of interaction between drugs and antibiotics, e.g. the aminoglycoside antibiotic gentamicin forms complex with promethazine. According to Eckert et al. [20], the complex was identified as electron charge transfer complex.

The charge transfer complex of clozapine may have altered pharmacological activities compared to the single drug [14].

Since the superdelocalizability of pi-electrons can be the basis of charge transfer complex formation, the results show the importance of CT-complex (charge transfer) formation in the binding of clozapine to the receptors in human brain and in the bacterial plasmid replication system.

Plasmid elimination also can be due to gyrase enzyme inhibition in plasmid replication machinery [21], where the drug binds to the receptor via biological charge transfer formation [18].

Our results show that there is an interaction between clozapine and penicillins. It is supposed that this interaction can have some clinical importance. In the best case, we suggest that the regular dose of clozapine can be reduced in the patients during their penicillin treatment. Further questionable possibility is that the penicillin can be administered together with clozapine in order to increase the neuroleptic effect of the latter [14].

Harmful in vivo interactions between the two medicines can be overcome by the withdrawal of clozapine. Moreover, clozapine should not be recommended during essential penicillin treatment [14]. However, there is of course a possibility that ampicillin administered together with clozapine may induce changes at the drug receptor site or in some of the biochemical processes that should lead to the enhanced neuroleptic effects of clozapine in the patients [14].

Since clozapine exerted a haemolytic effect on blood agar, the harmful side effects of clozapine should also be considered and studied in vivo. In patients with psychotic symptoms who had failed to improve on clozapine treatment, improvements were achieved with electroconvulsive therapy (ECT). It was reported by Gujavarty and co-workers [22], that a combination of electroconvulsive therapy and clozapine increased the efficacy of antipsychotic action because of the effect of ECT on the permeability of the blood-brain barrier. Considering this study, we may conclude that ampicillin can also increase the permeability of clozapine on the blood-brain barrier. There is another possibility, i.e. the charge transfer complex of clozapine and penicillins has a better permeability through the blood brain-barrier. The possible

beneficial and harmful effects of this drug interaction need further investigation both in vivo and in vitro.

Acknowledgements. This work was supported by the COST ACRIVAL Programme No. 815 the Commission of the European Communities, Grant No. ERBCIPECT 926043 and Grant OTKA 2703 from the Hungarian Research Found.

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RESISTANCE TO BETA-LACTAM ANTIBIOTICS AND BETA-LACTAMASE PRODUCTION OF *BACTEROIDES*, *PORPHYROMONAS* AND *PREVOTELLA* STRAINS*

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(Received November 3, 1994)

(Accepted December 9, 1994)

Resistance to beta-lactam antibiotics of 183 clinical isolates belonging to *Bacteroides*, *Porphyromonas* and *Prevotella* was tested by a micro-broth dilution MIC method. Beta-lactamase production was screened by using nitrocefin sticks. Prevalence of resistance to different beta-lactam antibiotics was higher among the *Bacteroides* strains other than *B. fragilis* parallel with a beta-lactamase production (88% and 96%, respectively). Resistance was observed less frequently among *Porphyromonas* and *Prevotella* strains. Cefoxitin resistance was 11.5% and amoxicillin/clavulanic acid 3.5% among *Bacteroides* isolates, whereas no resistance was found to these antibiotics among the *Porphyromonas* and *Prevotella* strains. All strains tested were susceptible for imipenem. Beta-lactamase production of selected isolates was tested quantitatively. Beta-lactamase of *B. fragilis* 1 and *B. levii* differed in their isoelectric points, substrate profiles and inhibition by clavulanic acid, sulbactam and tazobactam.

Gram-negative anaerobic bacteria are well recognised pathogens in various human infectious. The most frequent isolates are the different species of genus *Bacteroides* [1], *Porphyromonas* and *Prevotella* after abdominal, pelvic or oral surgery. Most anaerobic bacteria involved in human diseases are still susceptible to antimicrobial agents used in the treatment and prophylaxis of anaerobic infections.

*This paper is dedicated to Professor Ilona Béládi, director emeritus of the Institute of Microbiology of Albert Szent-Györgyi University Medical School in Szeged, Hungary, by her pupils on the occasion of her 70th anniversary.

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However, during the past several years increasing number of reports in the literature indicated that a great proportion of anaerobic isolates, especially members of the genus *Bacteroides*, *Porphyromonas* and *Prevotella* are becoming resistant, first of all to beta-lactam antibiotics [2, 3, 4].

For anaerobic and facultatively anaerobic bacteria three mechanisms of resistance to beta-lactam antibiotics are known: alteration in the number or type of penicillin binding proteins, alteration of the outer membrane pores or porins resulting in blocking of the penetration of drugs, and the most frequently observed mechanism, production of beta-lactamases with different activity to hydrolyse the various beta-lactam antibiotics [2]. In *Bacteroides*, *Porphyromonas* and *Prevotella* strains resistance to beta-lactam antibiotics is most commonly mediated by the production of beta-lactamases [5]. The high percentage of ampicillin-resistant strains among clinical isolates of *Bacteroides*, *Porphyromonas* and *Prevotella* strains during routine testing of susceptibility (data to be submitted for publication) prompted us to investigate the resistance of these isolates, collected in three centres in Hungary, to a wide range of beta-lactam antibiotics and to study their beta-lactamase production.

Materials and methods

Bacterial strains. A total of 183 clinical isolates originated from severe infectious after abdominal, gynaecological or oral surgery were involved in this study. The strains were isolated between June 1992 and July 1993. Identification of the strains was carried out according to the guidelines of the VPI manual [6] and the Manual of Clinical Microbiology of the American Society of Microbiology [7]. Gas-liquid chromatography was used for further characterization of the strains (Simadzu GC-14A, Japan). All the plates were incubated in an anaerobic chamber (Bactron, Shel Lab, England) or in the GasPack (Oxoid) system.

Testing of antibiotic resistance. MIC determination of the beta-lactam antibiotics was carried out by the micro-broth dilution method according to the guidelines of NCCLS [8]. The microtiter plates containing the two-fold dilution of the antibiotics in Wilkins-Chalgren broth were prepared in our laboratory. The plates were maintained at -20°C until use. Before inoculating the plates, they were kept at room temperature for 15 min. The microtiter plates were incubated in an anaerobic chamber for 48 h. The MIC values of the antibiotics were read with help of a mirror. In the case of a limited number of strains the "E" test (Biodisk) was also applied for determination of the MIC values of ampicillin, cefoxitin, amoxicillin/clavulanic acid and imipenem parallel with the micro-broth dilution method. *B. fragilis* ATCC 25285 was used as a control strain.

Determination of the beta-lactamase activity: Screening for beta-lactamase activity of all strains was carried out by use of nitrocefin sticks (Oxoid) according to the instructions of the manufacturer. Colonies of 48 h cultures were touched by the nitrocefin stick. The colour change from yellow to red was evaluated within 5 min. The enzyme activity of selected strains was determined quantitatively. Crude enzyme extracts were prepared from 48 h BHI broth cultures of the strains. After centrifugation at 10 000 g for 30 min at 4°C the pellet was resuspended in 0.05 M phosphate buffer (pH 7.0) to the original volume then ultrasonicated. The culture supernatants and the supernatants of the ultrasonicated cells were used in quantitative assays where nitrocefin (100 μM) in 0.05 M phosphate buffer (pH 7.0) was used as substrate and the reactions were measured spectrophotometrically [9]. All determinations were carried out in duplicates. One unit of beta-lactamase was defined as the amount which formed 1.0 μM of product per minute under these conditions.

Stability studies. The crude enzymes were stored at different temperatures; -70, -20, 4 and 18 °C for 7 days. The remaining beta-lactamase activity was measured after 48 h and 7 days by the nitrocefin assay.

Inhibition studies. Inhibition of the beta-lactamase activity was determined with nitrocefin as substrate in the spectrophotometric assay. Clavulanic acid, sulbactam and tazobactam in various concentrations were preincubated together with the beta-lactamases of the *B. fragilis* 1 and the *B. levii* strains for 30 min at 30 °C (0.05 M phosphate buffer, pH 7.5) before determination of the remaining beta-lactamase activity. Percentage inhibition was calculated as $100 \times [(c-r)/c]$, where *c* is the activity in control samples incubated without inhibitor and *r* is the remaining activity in samples incubated with the inhibitors [9].

Substrate profile. Substrate profile of beta-lactamases obtained from the strains *B. fragilis* 1 and *B. levii* were determined. Penicillin G, ampicillin, piperacillin, cephalotin, cefoxitin and imipenem were used as substrate. The K_m and V_{max} values were determined in a competitive assay where the hydrolysis rate of nitrocefin was measured with or without the antibiotics [9].

Isoelectric focusing. In the case of selected strains the isoelectric focusing of the beta-lactamases was carried out with commercially available ampholine gels providing a pH gradient of 4.0 to 6.5 (Pharmacia). Demonstration of the beta-lactamase activity in the gels was achieved with nitrocefin as a substrate.

Results

Altogether 183 strains belonging to *B. fragilis* (82), other *Bacteroides* species (64), *Porphyromonas* (15) and *Prevotella* (22) species were collected during a period of one year in three centres in Hungary (Szeged, Veszprém, Salgótarján). Table I shows the percentage of resistant strains to a wide range of beta-lactam antibiotics according to the breakpoints suggested by the NCCLS [8]. *B. fragilis* and the other *Bacteroides* spp. showed much higher resistance to the different beta-lactam antibiotics than the *Porphyromonas* and *Prevotella* species tested, however 47% of the *Porphyromonas* and 36% of the *Prevotella* strains proved to be resistant to penicillin. The *Bacteroides* strains other than *B. fragilis* showed higher resistance rate to some of the beta-lactam antibiotics such as ampicillin, carbenicillin, piperacillin and moxalactam. Altogether five *Bacteroides* strains (5% and 2% of *B. fragilis* and other *Bacteroides*, respectively) were resistant to amoxicillin/clavulanic acid. No imipenem resistant strain was found during this study. No differences were observed between the strains collected in different areas of Hungary. Results obtained by the "E" test showed identical results within ± 1 twofold dilution for ampicillin, cefoxitin and imipenem. Major discrepancies were observed for amoxicillin/clavulanic acid, but they did not influence the ranging of the strains in susceptibility categories.

When the beta-lactamase production of the strains was screened by the nitrocefin stick method, 96% of the *B. fragilis*, 88% of the other *Bacteroides* species proved to be positive. Much lower incidence of beta-lactamase production was observed among *Porphyromonas* and *Prevotella* strains, 47% and 36%, respectively (Table II), parallel with the lower percentage of the strains resistant to beta-lactam antibiotics (Table I). The incidence of high level resistance to beta-lactam antibiotics used most frequently in treatment of infections involving anaerobic bacteria was observed among *B. fragilis* and other *Bacteroides* species (Table III). Twelve of 82 *B.*

fragilis strains and 8 of 64 strains belonging to different *Bacteroides* species other than *B. fragilis* showed high level resistance to ampicillin, mezlocillin and piperacillin (MIC>128 mg/l), while eight *B. fragilis* and five other *Bacteroides* sp. strains were resistant to cefoxitin and moxalactam (MIC>64 mg/l). Only 5 strains proved to be resistant to amoxicillin/clavulanic acid (MIC>8 mg/l). Cross-resistance to ten selected beta-lactam antibiotics was evaluated for the *Bacteroides* isolates (Table IV). High percentage of the strains which were resistant to carbenicillin, mezlocillin, cefoxitin, moxalactam and piperacillin, showed cross-resistance with other beta-lactam antibiotics. All five strains, which proved to be resistant to amoxicillin/clavulanic acid were also resistant to the other antibiotics tested except cefoxitin for which two strains were susceptible.

Low percentage of the strains exhibited resistance to the other beta-lactam antibiotics in the group of strains with penicillin or ampicillin resistance. None of the strains resistant to any beta-lactam antibiotics was resistant to imipenem. All but five strains which were resistant to different beta-lactam antibiotics were susceptible to amoxicillin/clavulanic acid.

Table I

Percentage of resistant *Bacteroides*, *Porphyromonas* and *Prevotella* strains to beta-lactam antibiotics

Antibiotics	Break point of resistance (mg/l)	Percentage of resistant strains of			
		<i>B. fragilis</i> (82)	Other <i>Bacteroides</i> spp. (64)*	<i>Porphyromonas</i> spp. (15)**	<i>Prevotella</i> spp. (22)***
Penicillin	4	97	96	47	36
Ampicillin	32	36	45	13	18
Carbenicillin	128	20	25	20	32
Mezlocillin	64	18	16	20	32
Piperacillin	64	19	25	13	18
Amoxicillin / clavulanic acid	8	5	2	0	0
Cephalothin	32	98	97	13	18
Cefoperazone	32	67	62	13	32
Cefotaxime	32	66	67	13	18
Ceftazidime	32	62	67	20	18
Cefoxitin	32	12	11	0	0
Ceftriaxone	32	8	11	0	0
Moxalactam	32	15	17	0	0
Imipenem	4	0	0	0	0

* see Table

** 3 *P. asaccharolytica*, 12 *P. gingivalis*

*** 5 *P. bivia*, 7 *P. disiens*, 18 *P. intermedia*, 2 *P. leoechei*

Table II

Frequency of beta-lactamase production in *Bacteroides*, *Porphyromonas* and *Prevotella* strains tested by nitrocefin stick (Oxoid)

Species	No. of strains tested	No. of beta-lactamase producing strains (%)
<i>B. fragilis</i>	82	79 (96)
Other <i>Bacteroides</i> spp.	64	56 (88)
<i>Porphyromonas</i> spp.	15	7 (47)
<i>Prevotella</i> spp.	22	8 (36)

Table III

Prevalence of high level resistance against selected beta-lactam antibiotics in different species of the *Bacteroides* genus

Species	No. of strains	No. of strains with		
		MIC>128 mg/l AMP, MEZ, PIP	MIC>64 mg/l CEF, MOX	MIC>8 mg/l A/C
<i>B. fragilis</i>	82	12	8	4
<i>B. ovatus</i>	9	2	2	0
<i>B. vulgatus</i>	20	1	1	0
<i>B. distasonis</i>	17	0	0	0
<i>B. thetaiotaomicron</i>	13	4	1	0
Other				
<i>Bacteroides</i> spp.*	5	1	1	1

AMP=ampicillin, MEZ=mezlocillin, PIP=piperacillin, CEF=cefoxitin, MOX=moxalactam, A/C= amoxicillin/clavulanic acid

* 4 *B. splanchnicus*, 1 *B. levii*

Ten strains, nine *Bacteroides* and one *Porphyromonas* isolates were selected for further studies. These strains showed high-level resistance (MIC>256 mg/l) for a wide range of beta-lactam antibiotics (Table V). They were all susceptible to imipenem and all but two strains to amoxicillin/clavulanic acid. As shown in Table VI, beta-lactamase activities were measured in the culture supernatants and in the supernatants of the ultrasound treated cells (crude enzyme extract). A negligible amount of enzyme could be detected in the culture supernatants of all strains.

Table IV

Cross-resistance to different beta-lactam antibiotics among *Bacteroides* strains

Antibiotics (No. of strains resistant)	Percentage of strains resistant to									
	PEN	AMP	A/C	CARB	MEZ	CTX	CXT	MOX	PIP	IMP
Penicillin (141)	100	61	3	21	18	61	22	17	19	0
Ampicillin (60)	100	100	10	16	34	75	28	31	37	0
Amoxicillin / clavulanic acid (5)	100	100	100	100	100	100	60	100	100	0
Carbenicillin (32)	97	97	16	100	81	91	63	60	81	0
Mezlocillin (25)	100	100	20	87	100	94	67	70	94	0
Cefotaxim (97)	87	86	5	32	26	100	18	18	26	0
Cefoxitin (17)	100	100	17	100	90	100	100	76	88	0
Moxalactam (23)	96	93	21	78	82	88	67	100	78	0
Piperacillin (32)	100	100	16	76	76	68	62	62	100	0
Imipenem ()										0

Table V

MIC values of beta-lactam antibiotics against 10 selected *Bacteroides*/*Prevotella* strains*

Strains	MIC (mg/l)					
	A/C	CFP	CTX	CXT	MOX	IMP
<i>B. fragilis</i> 1	128	256	256	128	64	4
<i>B. fragilis</i> 2	4	256	256	32	64	2
<i>B. fragilis</i> 3	8	>256	256	32	16	0.5
<i>B. vulgatus</i> 1	64	128	>256	32	32	0.25
<i>B. vulgatus</i> 2	4	256	128	32	32	0.25
<i>B. distasonis</i>	2	256	>256	128	64	1
<i>B. ovatus</i>	8	>256	256	256	128	4
<i>B. thetaiotaomicron</i>	4	256	256	64	>256	4
<i>B. levii</i>	8	>256	256	8	8	0.25
<i>P. oralis</i>	8	128	256	64	256	<0.25

* All strains were resistant to penicillin G, ampicillin, mezlocillin, carbenicillin and cephalotin with a MIC>256 mg/l

A/C=amoxicillin/clavulanic acid, CFP=cefoperazone, CTX=cefotaxime, CXT=cefoxitin, MOX=moxalactam, IMP=imipenem

The amount of the cell bound enzyme varied between 0.18 and 1.36 U/ml measured spectrophotometrically with nitrocefin as substrate. The isoelectric points of four beta-lactamases tested ranged between pH 4.3 to 5.1 (Table VI).

Table VI

Characterization of beta-lactamases from 10 selected *Bacteroides/Prevotella* strains

Strains	Enzyme activity/ml*		Isoelectric point (pH)
	Culture supernatant	Ultrasonicated cells	
<i>B. fragilis</i> 1	< 0.01	1.36	4.6
<i>B. fragilis</i> 2	0.05	0.19	nt
<i>B. fragilis</i> 3	< 0.01	0.23	nt
<i>B. vulgatus</i> 1	< 0.01	0.45	4.6
<i>B. vulgatus</i> 2	0.08	0.18	nt
<i>B. distasonis</i>	0.01	0.20	nt
<i>B. ovatus</i>	0.03	0.48	4.3
<i>B. thetaiotaomicron</i>	0.05	0.36	nt
<i>B. levii</i>	0.01	0.96	5.1
<i>P. oralis</i>	0.02	0.28	nt

* μ M of nitrocefin degraded per minute

nt = not tested

The inhibition experiments and the substrate profile determination were carried out for the two most active beta-lactamases. Under the same experimental conditions, 40 μ mol/ml sulbactam effectively inhibited the activity of the beta-lactamase isolated from a *B. levii* strain, whereas it was completely ineffective against the beta-lactamase isolated from the *B. fragilis* strain (Fig. 1). The inactivation of the *B. levii* beta-lactamase by sulbactam was concentration-dependent (Fig. 2). On decrease of the sulbactam concentration to 0.4 μ mol/ml, the inhibitor was found to be totally inactive. The two other beta-lactamase inhibitors also exhibited different effects on the two beta-lactamases. At the same concentration (40 μ mol/ml), they were less effective against the *B. fragilis* beta-lactamase than against the *B. levii* enzyme (Fig. 3). The same differences were seen when the various concentration of the three beta-lactamase inhibitors were incubated for 30 min together with the partly purified beta-lactamases originating from the two *Bacteroides* strains and the enzyme activities were determined with nitrocefin as substrate. For sulbactam, clavulanic acid or tazobactam, decrease of the concentration resulted in a significantly lower inhibition of the *B. fragilis* beta-lactamase than that originating from the *B. levii* strain (Fig. 4).

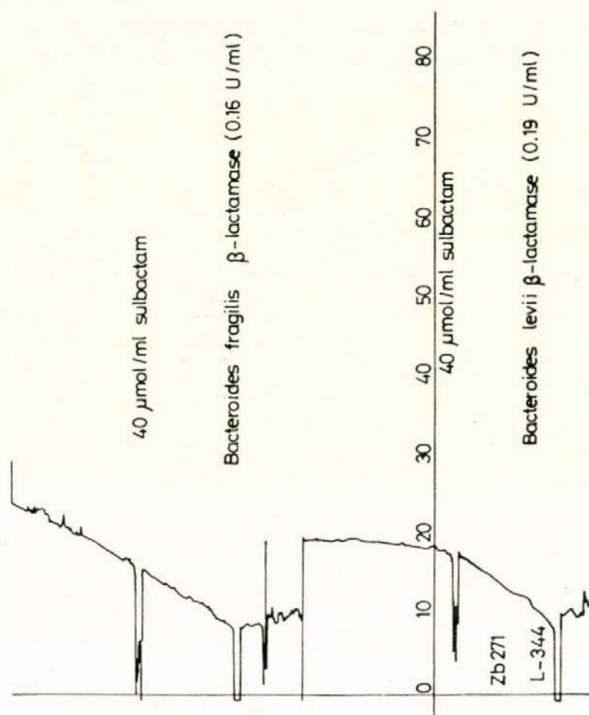


Fig. 1. Inactivation by sulbactam of beta-lactamases isolated from *B. levii* and *B. fragilis*

The *B. levii* beta-lactamase was stable when incubated at different temperatures for 48 h or 7 days. The *B. fragilis* beta-lactamase was also stable at room temperature, but only 52–85% of the original activity was found when it was maintained at 4, –20 or –70 °C for 48 h. Only negligible changes were seen in the activities after 7 days incubation. The use of 1% glycerol did not improve the stability of the enzyme.

The K_m values and the relative rates of hydrolysis of the different substrates, expressed as a percentage of the hydrolysis of the penicillin G, are shown in Table VII. These data again reveal differences between the two enzymes. The relative rates of hydrolysis of cefalothin, ampicillin and piperacillin were higher than that observed with penicillin G as substrate. Cefoxitin was hydrolysed only by the *B. fragilis*.

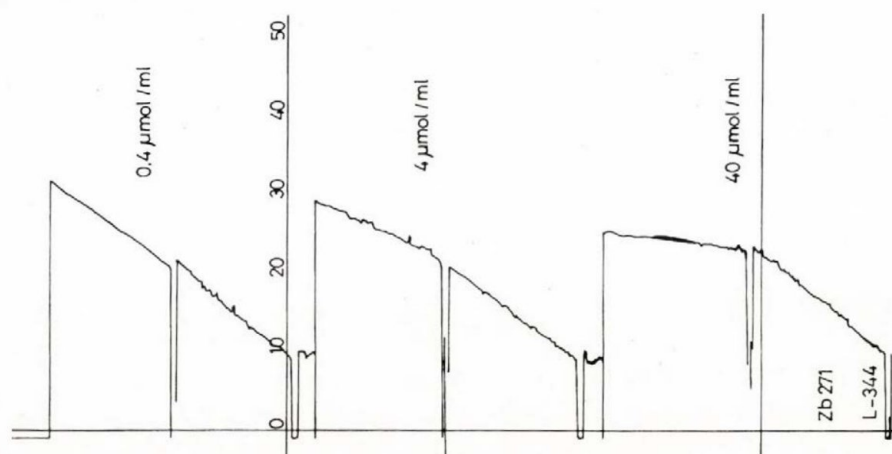


Fig. 2. Inactivation by different concentrations of sulbactam of beta-lactamases isolated from *B. levii*

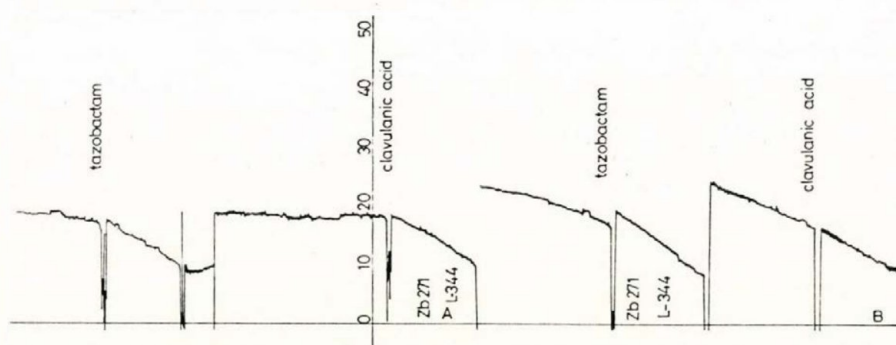


Fig. 3. Inactivation by clavulanic acid (40 µmol/ml) and tazobactam (40 µmol/ml) of beta-lactamases isolated from *B. levii* (A) and *B. fragilis* (B)

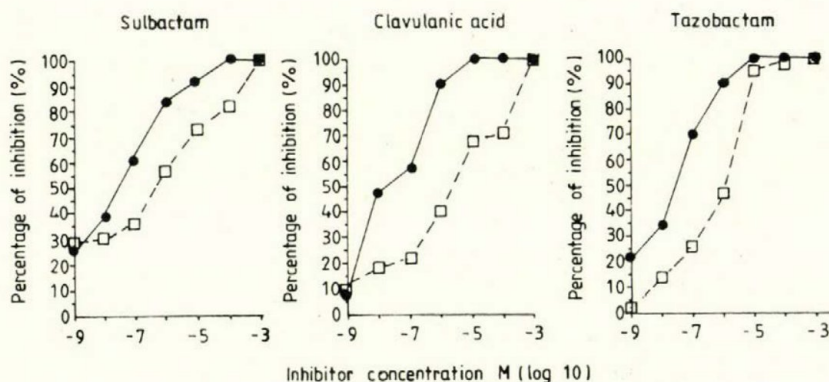


Fig. 4. Effects of different concentrations of three beta-lactamase inhibitors on activity of beta-lactamases isolated from *B. fragilis* (□ ---- □) and *B. levii* (● ---- ●)

Discussion

Anaerobic bacteria are important pathogens of man causing a variety of infections. *B. fragilis* and the related species are the most frequent anaerobic isolates in mixed infection as intestinal contaminants [10]. *Porphyromonas* and some *Prevotella* species (formerly members of both genera belonged to *Bacteroides*) are found frequently in the human oral cavity [10, 11]. Some of them are important pathogens in oral, dental and bite transmitted infections, but may produce infections also of the head, neck and lower respiratory tract. *Prevotella bivia*, *Prevotella disiens* and *Porphyromonas asacharolyticus* are also prevalent in urogenital and intestinal tracts and are important pathogens in gynecological infections [12]. *Bacteroides levii* is a pigmented *Bacteroides* species of animal origin which can be isolated rarely from human clinical specimens [7]. *Prevotella* and *Porphyromonas* isolates of the upper body sites were formerly considered to be sensitive to penicillin but resistance has increased [13]. Resistance is due to the production of beta-lactamase which (unlike in *B. fragilis*) hydrolyses penicillin more readily than cephalosporins [5, 14]. Resistance of *B. fragilis* and other members of genus *Bacteroides* to different beta-lactam antibiotics is well known, however the rate of resistance to some beta-lactams such as cefoxitin or moxalactam, considered resistant to beta-lactamases of *Bacteroides* strains, increased significantly during recent years [15, 16, 17]. In our study resistance to beta-lactam antibiotics was quite common among *B. fragilis* and other *Bacteroides* species. Resistance was less frequently observed among species belonging to *Porphyromonas* or *Prevotella*. In a study carried out in 1988–1989 by 22 laboratories in 15 European countries 1289 *Bacteroides* strains were tested for some beta-lactam antibiotics [18].

The overall resistance to ampicillin, mezlocillin, cefoxitin and ceftazidim of the *B. fragilis* strains were 12%, 6%, 2%, 12%, whereas that of the related *Bacteroides* strains 16%, 6%, 3%, 41%, respectively. A great difference was observed among the resistance data in different countries. Our data concerning ampicillin, mezlocillin, cefoxitin and ceftazidim resistance are very similar to those found in Spain [18, 19] or the data published recently from Korea [20]. Five of our *Bacteroides* strains (3.5%) proved to be resistant to amoxicillin/clavulanic acid compared with the 1% resistance observed in the European study. This also resembles the Spanish data (4%) [19]. None of the *Porphyromonas* and *Prevotella* strains showed resistance to amoxicillin/clavulanic acid. Resistance to carbenicillin, mezlocillin and piperacillin as well as to cefoperazone, cefotaxime, and ceftazidime were very similar among the *Bacteroides* isolates. Our *B. fragilis* strains and strains belonging to other *Bacteroides* spp. proved to be more susceptible to ceftriaxone than to any other cephalosporin tested (8 and 11%, respectively). No imipenem resistant strain was found during this study compared with the very low incidence of resistance (0.3%) observed in the European surveillance [18]. Resistance to carbapenems, exemplified by imipenem, is very rare, but a small number of resistant strains of *B. fragilis* group was reported in the United Kingdom, France, Sweden, Japan and the United States [21–23]. This resistance was also found to be due to beta-lactamase production, where the enzymes, similar to the carbapenemases produced by some aerobic bacteria (*Flavobacterium odoratum*, *Xantomonas maltophilia*) are metallo-enzymes [24]. Cross-resistance studies showed considerable differences between beta-lactam antibiotics. Penicillin, ampicillin and cefotaxime resistant strains showed a low percentage cross-resistance with the other beta-lactams, whereas carbenicillin, mezlocillin, cefoxitine, moxalactam and piperacillin resistant strains were resistant frequently to the other beta-lactam antibiotics as well (Table IV).

Table VII

Hydrolysis of various beta-lactam antibiotics by beta-lactamases from *B. fragilis* 1 and *B. levii*

Substrate	Hydrolysis by beta-lactamase from			
	<i>B. fragilis</i> 1		<i>B. levii</i>	
	K _m (μM)	V _{max} *	K _m (μM)	V _{max}
Penicillin G	6.2	100	9.5	100
Ampicillin	80	270	86	310
Piperacillin	420	630	7.2	120
Cefalothin	11	480	130	650
Cefoxitin	156	58	0.06	0.01
Imipenem	1.9	0.01	0.1	0.01

* Relative rates of hydrolysis of substrates are expressed as percentages of penicillin G hydrolysis

The usage of the nitrocefin containing sticks as screening method showed high percentage beta-lactamase production among *B. fragilis* and other *Bacteroides* strains (96% and 88%, respectively) in our study. Small amounts of constitutive beta-lactamase production can be found in almost 100% of *Bacteroides* strains [2, 5, 25]. The enzyme is cell bound and only negligible amount of the enzyme can be found in the culture supernatants (Table VI). Without extraction of the enzyme in those strains where the production is very low, the screening test may fail to detect it. Olsson et al. [14] showed that about 6% of the strains produce large amount of the enzyme. In our case out of 10 strains, selected according to their broad and high-level resistance to different beta-lactam antibiotics, only two produced relatively high amount of beta-lactamases (Table VI). We found significant differences between them according their substrate profile and inhibition by clavulanic acid, sulbactam or tazobactam. All three inhibitors were less effective on the beta-lactamase isolated from the *B. fragilis* than that of obtained from the *B. levii* strain. The *B. fragilis* strain was resistant to all beta-lactam antibiotics except imipenem, the MIC value of which being close to the breakpoint. The *B. levii* strain, resistant to a broad spectrum of beta-lactams, proved to be sensitive to cefoxitin, moxalactam and imipenem (Table V). Although *Bacteroides* strains studied in various laboratories may produce different beta-lactamases as judged by specific activity, substrate profile and isoelectric focusing, three types of the enzymes can be characterized [26]. (i) Strains, fully susceptible to moxalactam, cefoxitin and imipenem produce beta-lactamase with low specific activity and broad susceptibility to enzyme inhibitors including clavulanic acid. (ii) Strains which are resistant to, and slowly inactivate moxalactam and cefoxitin. They are less sensitive to imipenem but do not inactivate it. These enzymes can be inhibited by imipenem, and somewhat less effectively by moxalactam, cefoxitin, clavulanic acid and sulbactam. (iii) Strains that rapidly inactivate imipenem and moxalactam and more slowly cefoxitine. Beta-lactamase of our *B. levii* strain seems to belong to the first group of *Bacteroides* enzymes, whereas *B. fragilis* enzyme to the second group.

Differences in the resistance to beta-lactam antibiotics (especially to cefoxitin, moxalactam and amoxicillin/clavulanic acid) between laboratories and countries reported by different authors and observed during the European surveillance, may be accounted for by differences in antibiotic usage in humans. Differences in the resistance can be observed sometimes between laboratories of the same country as well. This was not the case this time in Hungary, as the strains were collected in three centres of the country and no significant differences were observed in their resistance to beta-lactam antibiotics (data not shown). Because of the differences in antibiotic susceptibility to beta-lactam antibiotics (as well as to other antibiotics) between different anaerobic bacteria, sometimes between single isolates of the same species, it is difficult to predict the sensitivity of an individual strain isolated from severely ill patient. To screen anaerobic bacteria for antibiotic resistance to follow the development of resistance mechanisms among the most common anaerobic bacteria seems to be an important task of clinical microbiological laboratories.

Acknowledgement. This study was supported to some extent by the Hungarian Research Fund (OTKA 2698). Characterization of the beta-lactamases was partly carried out in the Department of Oral Microbiology, Karolinska Institute, Stockholm, Sweden, supported by the Swedish Institute. Part of this study was presented during the 18th International Congress of Chemotherapy in Stockholm.

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EFFECTS OF PENTOXIFYLLIN AND PENTAGLOBIN[®] ON TNF AND IL-6 PRODUCTION IN SEPTIC PATIENTS*

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(Received November 3, 1994)

(Accepted November 10, 1994)

Pentoxifylline inhibited the TNF production of purified human white blood cells and whole blood cultures stimulated either by LPS or by *Staphylococcus aureus*. PTX did not influence the CD14 expression. The in vitro TNF and IL-6-producing capacities of septic patients were higher than in the control group. Administration of PTX to septic patients resulted in the normalization of TNF synthesis and in a moderate decrease in IL-6 production. It also subsequently led to an improvement in the clinical status. A further improvement in APACHE II score could be achieved by administration of Pentaglobin[®] (Biotest). The prevention of in vitro TNF production by Pentaglobin[®] could be demonstrated involving the use of whole blood rather than purified lymphocytes. The level of soluble ICAM-1 in the serum of septic patients was significantly higher than in normal individuals, but it decreased following PTX and Pentaglobin[®] therapy. It is presumed that PTX and Pentaglobin[®] can antagonize cytokine production at different levels, resulting in synergistic action that is beneficial in the treatment of sepsis.

Septic shock is a catastrophic consequence of invasive infection. Unfortunately, recent advances in surgery and medicine have not reduced significantly the overall mortality from septic shock. It is obvious that TNF has a key position in the pathogenesis of septic shock [1]. Suppression of its biosynthesis therefore might be beneficial in the treatment of sepsis and septic shock. The aim of our study was to

*This paper is dedicated to Professor Ilona Béládi, director emeritus of the Institute of Microbiology of Albert Szent-Györgyi University Medical School in Szeged, Hungary, by her pupils on the occasion of her 70th anniversary.

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investigate the effects of pentoxifyllin (PTX) and Pentaglobin[®] an IgM-enriched immunoglobulin preparation on cytokine production in vitro and in septic patients in clinical practice.

Materials and methods

Patients. The study group comprised 31 patients with sepsis syndrome. The criteria for inclusion in the sepsis syndrome group included fever (temperature >38.3 °C) or hypothermia (<35.5 °C), tachycardia (>90/min), tachypnoea (>20/min), clinical evidence of infection, a leukocyte count greater than 12×10^9 /litre or less than 3×10^9 /litre, and at least one of the following: hypoxaemia (P_aO_2 <72 mmHg), oliguria (urine output <30 ml/h), unexplained metabolic acidosis or a recent change in mental status. Diagnoses included pancreatic abscess (n=28), or oesophageal and ventricular malignant tumour (n=3). The operation involved sequesterectomy combined with continuous washing and suction drainage, total oesophagectomy or gastrectomy.

Cells. Mononuclear cells were prepared by centrifugation of heparinized venous blood from healthy donors by density centrifugation on Ficoll-Paque (Pharmacia). Granulocyte-containing leukocytes were separated after sedimentation in 6% dextran solution. Monocytes were separated by incubation with immunomagnetic beads (Dynabeads, Dynal, Oslo) coated with anti-Leu-3 (CD14) mAb. Peripheral blood either from healthy donors or from septic patients, without separation, is referred to as whole blood cultures.

Induction of cytokine release. White blood cells (5×10^6 /ml) or whole blood cultures were incubated for 24 h at 37 °C with *Escherichia coli* LPS (SIGMA) at 50 µg/ml or with heat-killed *Staphylococcus aureus* (10^8 /ml), respectively. The supernatants were tested for the presence of TNF and IL-6.

TNF and IL-6 assays. TNF was titrated in a bioassay on cell line WEHI clone 13 [2]. IL-6 was measured via its proliferative action on the IL-6 dependent mouse hybridoma cell line B-9 [3]. The activities were calibrated against rh TNF (Amersham) or rh IL-6 (Amersham).

Reagents. Pentoxifylline (Trental[®], Hoechst) and Pentaglobin[®] (Biotest) were used in vitro experiments diluted with culture medium (RPMI 1640) or were administered intravenously at 200 mg/day or 5 mg/kg/day, respectively. Determination of sICAM-1 was performed by sICAM-1 ELISA (Bender Med Systems, Vienna), according to the instructions of the manufacturer.

FACS analysis. Monocytes were stained with FITC-conjugated CD14 monoclonal antibodies (Becton Dickinson), and fluorescence analysis was performed with a FACStar plus (Becton Dickinson).

Determination of plasma endotoxin level. Endotoxin assays were carried out with a Coatest Endotoxin Kit (Kabi Vitrum, Sweden).

Results

Effects of pentoxifyllin (PTX) on TNF and IL-6 production of mononuclear cells. PTX significantly decreased the TNF production of mononuclear cells in a dose-dependent manner when either LPS or *S. aureus* was the inducer. In contrast, there was only a moderate decrease in IL-6 production (Fig. 1)

Effect of pentoxifyllin on the expression of CD14 antigens. As CD14 molecules play key position as receptors of LPS binding proteins [4] the expression of these molecules on monocytes was investigated in the presence or absence of PTX. The results of FACS analysis demonstrate (Fig. 2) that the number of fluorescent cells and

the intensity of fluorescence are the same even on pretreatment of mononuclear cells with 200 µg/ml PTX for 1 h. Thus, we presume that the LPS binding site of the monocytes is not involved in PTX-induced suppression of TNF production.

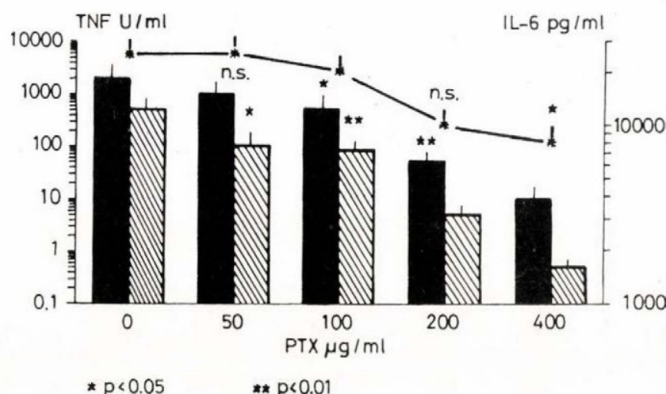


Fig. 1. Effects of pentoxifyllin on TNF and IL-6 production of mononuclear cells. Mononuclear cells (5×10^6 /ml) were induced with heat killed *S. aureus* (solid columns) or with 50 ng/ml *E. coli* (shaded columns) LPS. PTX was added together with the inducers. The TNF and IL-6 contents of the supernatants were determined after incubation for 24 h. Data are means $\pm$ SD of the results of five separate experiments

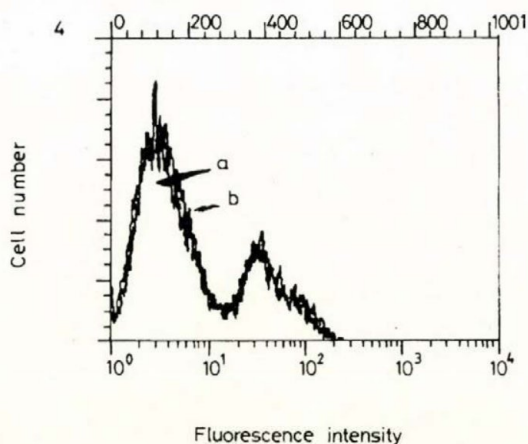


Fig. 2. Effects of pentoxifyllin on CD 14 antigen expression on monocytes. PTX-treated (a) and control (b) mononuclear cells were stained with FITC-conjugated CD14 MoAb, then analysed for fluorescence on a flow cytometer FACStar plus Becton Dickinson

Effects of pentoxifyllin on TNF production of different leukocyte populations. Comparison with effects of Pentaglobin^o. We investigated whether the inhibitory effect of PTX is specific for any type of white blood cells and/or monocytes. The latter were separated by means of immunomagnetic beads (Dynabeads) coated with anti-Leu M3 antibodies (Becton Dickinson). These cells retained their biological activity, as they produced TNF after in vitro stimuli (Fig. 3), which can be blocked with PTX. Monocyte-depleted mononuclear cells were able to produce TNF and this too was inhibited by PTX. Analogous results were obtained with a mixture of mononuclear cells and granulocytes. When whole blood cultures were applied, the effectiveness of the drug was reduced, very probably because part of the PTX was bound to the red blood cells. A somewhat opposite effect was observed when Pentaglobin^o was applied at a concentration of 50 µl/ml cell culture. The inhibition of TNF synthesis was more pronounced when this was investigated in whole blood cultures (Fig. 4), very probably because of the presence of certain serum factors. The results of these experiments suggest that PTX and Pentaglobin^o might have complementary effects with regard to the inhibition of cytokine production after bacterial stimuli.

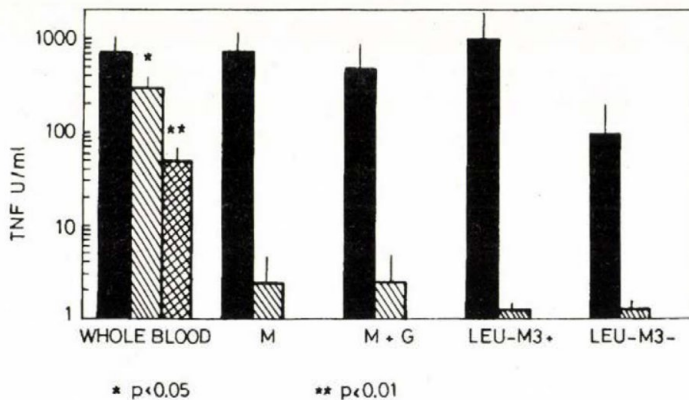


Fig. 3. Effects of pentoxifyllin (400 µg/ml - shaded columns; 800 µg/ml - hatched column; control - solid columns) on TNF production of different populations of white blood cells. Mononuclear cells (M), granulocytes and mononuclear cells (M+G) were separated as described in the Materials and methods. Monocyte-depleted mononuclear cells (Leu-M3-) were obtained after separation of monocytes (Leu-M3+) with Dynabeads. White blood cells and whole blood cultures were induced with *S. aureus*, and the TNF titres of supernatants were measured after incubation for 24 h. Data are means±SD of the results of three separate experiments

Beneficial effects of Pentoxifyllin and Pentaglobin^o in septic syndrome. PTX was administrated in a dose of 200 mg/day to septic patients (n=14) as a complement to conventional antibiotic therapy. This was supplemented with Pentaglobin^o (5

ml/body weight/day) for 3 subsequent days (n=6). The results were compared with those of earlier conventional intensive therapy without PTX or Pentaglobin[®] (n=11)

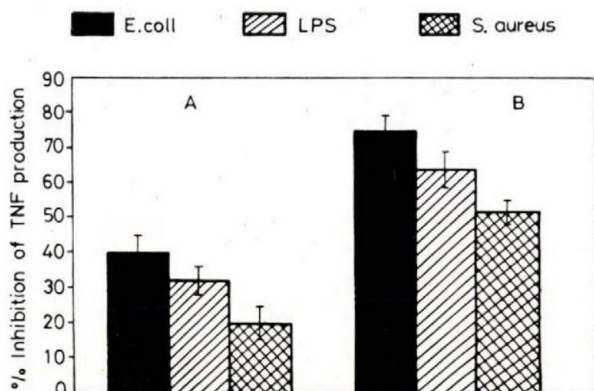


Fig. 4. Effects of Pentaglobin[®] on TNF production. Purified mononuclear cells (Exp. A) or whole blood cultures (Exp. B) of healthy donors were incubated with heat-killed *S. aureus* (hatched columns) or *E. coli* (solid columns) or *E. coli* LPS (hatched columns) (SIGMA) in the presence or absence of 50 μ l Pentaglobin[®]. The TNF contents of the supernatants were measured 24 h later. Data are means $\pm$ SD of the results of three experiments

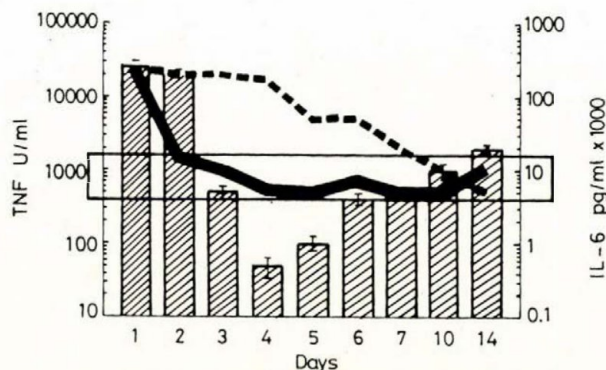


Fig. 5. Effects of pentoxifyllin on TNF and IL-6 production in septic patients. Whole blood cultures of septic patients were induced with *S. aureus*, and the TNF and IL-6 contents of the supernatants were determined. Hatched bars represent TNF production of septic patients without PTX treatment (n=11). Lines represent TNF production (continuous line) and IL-6 production (dotted line) in the PTX-treated group (n=14). Normal values (squared area) were obtained from healthy blood donors (n=15)

The kinetics of *in vitro* TNF and IL-6 production by the septic patients was determined (Fig. 5). The follow-up study in septic patients without PTX therapy ($n=11$) revealed that the *in vitro* TNF production was initially significantly higher than in the control group, but it decreased in the later phase of disease, in correlation with the severity of the disease, very probably as a consequence of the exhaustion of the white blood cells. This might sometimes be of prognostic value. The transient unresponsiveness to *in vitro* stimuli seemed to be reversible after the recovery. The same tendency, i.e. a biphasic response to *in vitro* stimuli was observed as concerns IL-6 production (data not shown in the Fig. 5). As a consequence of PTX therapy, the TNF production dropped to the control level on day 2. There was a moderate decrease in the level of IL-6 production.

sICAM-1 level in septic patients. A positive correlation was observed between the serum IL-6 level and the soluble ICAM-1 level in septic patients ($r=0.6837$, $n=49$, $p<0.001$, data not shown). The circulating sICAM-1 was significantly higher in septic patients (1000 ± 200 ng/ml) than in normal controls (250 ± 125 ng/ml), but it decreased slowly after PTX administration (Fig. 5). A more pronounced drop in serum ICAM-1 level was observed in those patients who additionally received Pentaglobin[®]. The severity of the illness was evaluated in accordance with the APACHE score system. Pentoxifyllin therapy resulted in a decreasing tendency in the scores, which tended to change inversely with the improvement in the clinical status, and the laboratory parameters. A further improvement was achieved in patients receiving both PTX and Pentaglobin[®] (Fig. 7). Figures 6 and 7 demonstrate only data on survivors in the Pentaglobin[®] group, because we lost two patients in consequence of surgical complications. The plasma endotoxin level in these four patients decreased from 40 ± 7.5 pg/ml to 2 ± 1.5 pg/ml (data not shown), whereas in the fatal cases the initial level (65 ± 8.5 pg/ml) decreased only transitorily and slightly and then returned to the original level in spite of administration for a subsequent 3 days (data not shown).

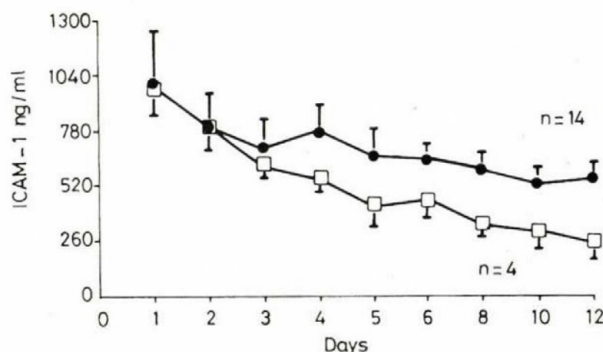


Fig. 6. Time course of sICAM-1 serum level in septic patients. Sera of patients receiving PTX (●) or PTX+Pentaglobin[®] (□) were collected on the days indicated, and the sICAM-1 concentration was determined by ELISA (Bender MedSystem). Value for normal donors was 200 ± 150 ng/ml ($n=10$), (data not indicated)

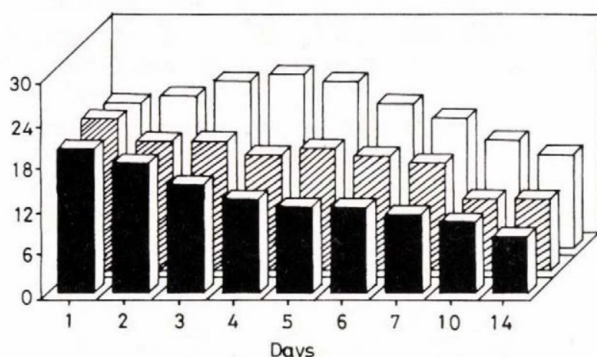


Fig. 7. Development of severity of septic disease as assessed by the Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system. Patients received PTX+Pentaglobin^o (n=4, solid columns), PTX (n=14, shaded columns); control patients (n=11) are indicated by open columns

Discussion

PTX is a well-known vasoactive drug with proven clinical efficacy in various circulatory disorders. Surprisingly it has been found that it is a potent inhibitor of TNF synthesis. Studies have clearly demonstrated that PTX blocks TNF mRNA production by macrophages [5]. It has been described [6] that PTX decreases the endotoxin-induced serum TNF level in human volunteers, whereas IL-6 are left were unaffected. This is in accordance with in vitro data that PTX does not affect IL-6 production [7]. In our studies, the production of TNF was inhibited by PTX not only after LPS stimuli but also after stimulation of effector cells by heat-killed *S. aureus*. However, IL-6 production was only slightly reduced, which was probably a consequence of the failure of the paracrine effect of TNF. This could be observed not only in the course of in vitro stimulation of mononuclear cells of healthy donors, but also during investigation of whole blood cultures of septic patients. The whole blood culture system for the induction of TNF and IL-6 seems to resemble closely the in vivo conditions. This method permits easy measurement of the cytokine producing capacity, and it is also convenient for repetition of measurements over long periods.

As Pentaglobin^o may interact with bacterial endotoxin and other bacterial antigens, it might prevent their stimulatory effect on cytokine producing cells. Thus, the combined application of Pentaglobin^o and PTX causes a greater suppression of TNF synthesis than can be achieved with either agent alone. Moreover the serum IL-6 level decrease was more pronounced in those patients who received Pentaglobin^o as well (data not shown), which correlated with the serum sICAM-1 level. Circulating or soluble intercellular adhesion molecule (sICAM-1) seems not to be directly related to

the underlying disease, but rather reflects a state of general cell activation, or can be regarded as a marker of the presence of inflammatory mediators and cytokines [8]. Our findings correlate well with the data of Küster and Degitz who described recently [9] the role of circulating ICAM-1 as an indicator in neonatal sepsis. The decrease in sICAM-1 was observed in parallel with the decrease in APACHE II score of our patients. However, in severe surgical complications (two fatal cases, with insufficient anastomoses and consecutive abscesses), even the combined treatment could not save the patient's life. This clearly demonstrates that only the influence of cytokine production and the optimal surgical and intensive care therapy together can result in the successful management of sepsis in surgical patients.

Acknowledgements. Supported by the Hungarian Research Found (OTKA 2727) and by the Hungarian Scientific Research Council, Hungarian Ministry of Health and Welfare (ETFT M-14). The excellent technical assistance of Mrs Györgyi Müller is gratefully acknowledged.

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THE ENTIRE *rne* GENE: THE REMAINING QUESTIONS*

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(Received November 3, 1994)

(Accepted December 1, 1994)

A 30-fold overexpression of the entire *rne* gene results in a 3-fold increase in RNase E activity. The overexpression of cloned *rne* fragments suggests that synthesis of the Rne protein is autoregulated and this regulation is related to the RNase E activity of the gene product. The enzyme produced by the cloned gene has an enhanced thermolability. The thermal stability of the temperature-sensitive enzyme increases when cells are cultivated in the presence of chloramphenicol, which suggests posttranslational alterations.

RNase E was earlier described as an RNA-processing enzyme of *Escherichia coli* responsible for the maturation of 5S rRNA [1]. It takes part in the copy control of *colE1* plasmids [2] and has a general role in the mRNA metabolism [3]. The *rne/ams* gene (the gene for RNase E) has been shown to be identical to *hmp1*, which encodes a 114 kD protein that migrates as a 180 kD protein [4]. A functional interaction exists between heat shock protein GroEL and an RNase E-like activity in *E. coli* [5]; *rne* is 32% homologous to a gene coding for 2-5 A-dependent RNase in mouse [6], which is a mediator of interferon action.

We previously reported that a high overproduction of RNase E resulted in only a 2–3-fold increase in enzymatic activity; the cloned enzyme was more temperature-sensitive than that coded by the chromosome, and RNase E was more thermally stable after chloramphenicol treatment of the cells suggesting posttranslational alterations [7–8].

One possible explanation of these puzzling observations was the finding that we cloned only part of the full *rne* gene, which still produced functional RNase E. The present experiments were carried out with plasmids containing the entire *rne* gene.

*This paper is dedicated to Professor Ilona Béládi, director emeritus of the Institute of Microbiology of Albert Szent-Györgyi University Medical School in Szeged, Hungary, by her pupils on the occasion of her 70th anniversary.

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Materials and methods

Strains and plasmids. Strains used: N3438, RNase E^{ts} (*rne-3071 recA*); N5713 (for preparing 7S RNA) [9]; HB101 [10]. Plasmids used: pGP1-4 contains the T7 RNA polymerase gene and is similar to pGP1-2 [11]; pT7-5 is similar to pT7-1 and both are used for cloning DNA fragments next to a T7 promoter in order to overexpress cloned DNA [11]; pRE107 [7] contains a 6 kb *Pst*I fragment from the *E. coli* chromosome which includes the *rne* gene; pRE141 is pT7-5 with a 3.2 kb *Cla*I-*Bam*HI fragment from the *rne* gene; pRE155 contains a smaller (1.9 kb) fragment, which is insufficient to express an active RNase E; pRE171 contains the 6 kb *Pst*I fragment from pRE107 in pT7-5.

For overexpression of the *rne* gene, we used established procedures [11, 12].

Cells were opened by sonication [8], and electrophoresis was carried out according to Laemmli [13].

Determination of RNase E activity. 7S RNA ($1-3 \times 10^3$ cpm) [9] was incubated in a reaction mixture (15 μ l) containing 10 mM Tris-HCl (pH 8.0), 100 mM NH₄Cl, 0.1 mM Na₂EDTA, 5 mM magnesium acetate and about 10 μ g protein (cell extract). To determine the thermal sensitivity of RNase E, cell extracts were preincubated at 43 °C for 30 min. Reaction mixtures were incubated at 30 °C. Incubation was terminated by addition of 5 μ l of a solution containing 50 mM Na₂EDTA, 1% SDS, 50% glycerol and 0.01% bromophenol blue. Samples were incubated in boiling water for 2 min and subjected to electrophoresis in a 5%/12% tandem polyacrylamide gel containing 7 M urea. For quantitation, after autoradiography, bands were excised from the dried gel and counted.

Results

As can be seen in Fig. 1, lane 4, when a plasmid containing the entire *rne* gene was overexpressed, the slowest migrating protein in the gel (about 180 kD) was amplified. The level of the Rne protein in lane 4 is about 30 times greater than that in lane 1 (the control, this is the level of Rne in the cell). Lane 2 shows the expression of a shorter insert coding for an active RNase E (pRE141). In this lane, the amount of the large band is very small. While the overexpression of a smaller insert (pRE155) does not inhibit synthesis of the large band, pRE155 codes for a protein, 55 kD in size, which does not have RNase E activity.

The RNase E activity of the extract made from cells overexpressing RNase E 30-fold was only 3 times higher than that of the control extract (Fig. 2). We previously found that the RNase E activity in a strain carrying plasmid pRE141 is more thermolabile in vitro than the chromosomally produced RNase E [8]. In vivo, cells containing pRE141 grow at 43 °C. Since we realized that we had used a deletion, it was possible that the RNase E activity produced by an incomplete protein is thermolabile. We therefore compared the RNase E in extracts prepared from a wild-type strain with extracts of a similar strain overexpressing the complete Rne protein.

As can be seen in Fig. 2, the RNase E activity expressed in a strain containing an *rne*⁺ gene in a plasmid is more thermolabile than the activity produced by the chromosomal gene.

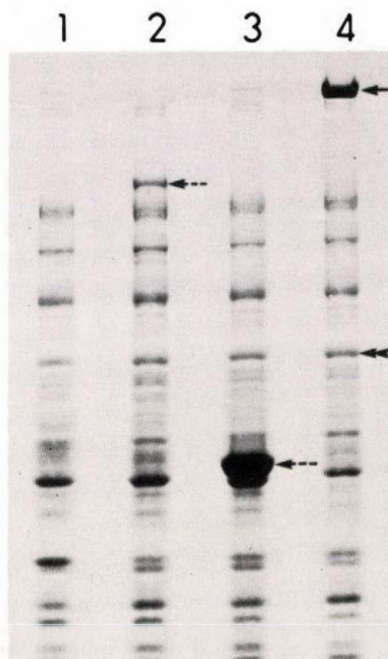


Fig. 1. Overexpression of RNase E. 5%/8% SDS-polyacrylamide gel stained with Coomassie Brilliant Blue. Only the 8% portion is shown. All four lanes contained extracts from strain HB101, which carries a plasmid containing the T7 RNA polymerase gene. In addition, each strain carried a second plasmid: pT7-5 (vector), lane 1; pRE141, lane 2; pRE155, lane 3; and pRE171, lane 4. The top arrow indicates the complete *rne* gene product (about 180 000 D, as determined by using protein markers). The gel was scanned, and the Rne bands were quantitated and normalized to a constant band (double arrow). The arrows to the right of lanes 2 and 3 indicate products from partially deleted *rne* genes. The level of the Rne protein in lane 4 was about 30 times greater than the level of that in lane 1

If protein synthesis is blocked by the addition of chloramphenicol to the culture, the RNase E activity produced in a strain containing the pRE141 plasmid is less thermolabile than before the addition [8]. We carried out similar experiments with the strain carrying plasmid pRE171 that produces the complete Rne protein, and again (Fig. 3) found that the resulting activity is less thermolabile.

The experiment was repeated with an RNase E^{ts} mutant (N3438) without plasmid. Figure 4 shows that in the presence of chloramphenicol the RNase E activity is more thermally stable (and higher).

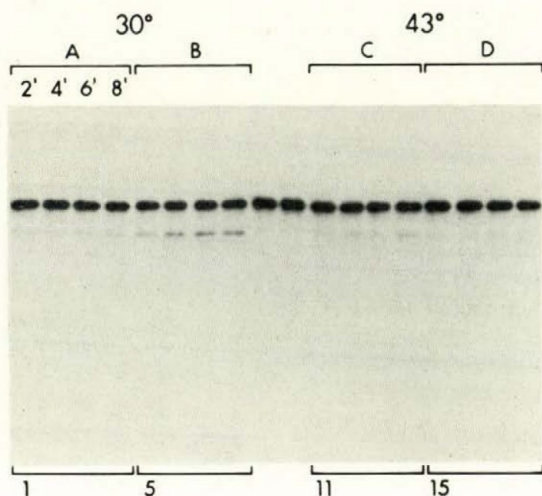


Fig. 2. RNase E expressed from pRE171 is more thermolabile than the enzyme coded by the chromosomal gene. Cell extracts were prepared from HB101 (*me*⁺) containing pT7-5 (control, lanes 1–4, A; and 11–14, C) or pRE171 (lanes 5–8, B; and 15–18, D). RNase E activity was assayed by using 7S RNA as substrate at 30 °C for the indicated times and the extracts were incubated at 30 °C (first 8 lanes; A, B) or 43 °C (last 8 lanes; C, D) for 30 min before the assay. Each assay contained 10 µg of cell extract prepared by sonication. The substrate was incubated alone at 30 °C for 2 (lane 9) or 6 (lane 10) min. An autoradiogram is shown from a 5%/12% polyacrylamide gel with 7 M urea

Discussion

The present studies show that cloning of the complete *rne* gene does not help to explain the puzzling observations found with shorter fragments: the cloned gene is temperature-sensitive in vitro, the temperature-sensitive enzyme is thermally more stable after chloramphenicol treatment of the cells, and overproduction of the Rne protein does not result in a similar increase in RNase E activity. Comack et al. [14] reported 50- to 60-fold increase in RNase E activity after overproduction. In their experiments they assayed a semi-purified preparation. The possibility, that an inhibitor prevents the enzyme activity in the crude extract cannot be excluded. Relatively large deletions in the *rne* gene do not result in loss of the RNase E activity.

Similarly, there are many recent observations which are not easy to explain. Casaregola et al. [15] demonstrated that the 180 kD Hmpl protein cross-reacted with antibodies raised against yeast heavy chain myosin, but they could detect no homology with myosin genes in the *ams/hmpl/rne* sequence [4]. Sohlberg et al. [5] precipitated RNase E activity with an antibody against GroEL, and detected RNase E activity in a highly purified GroEL preparation.

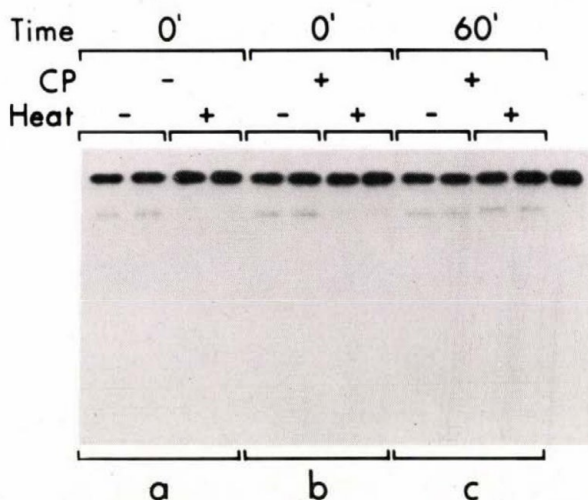


Fig. 3. Effect of chloramphenicol treatment on thermal sensitivity of RNase E activity. Extracts were prepared from a strain that contains the *rne-3071* ts mutation in the chromosome (N3438) and an *rne*⁺ plasmid (pRE171). Extracts were prepared from cells grown in the absence of chloramphenicol (CP) (panel a), or in the presence of 200 µg/ml chloramphenicol (b, c). At the indicated times, cells were harvested and extracts were prepared. They were assayed in duplicates for RNase E activity, using 7S rRNA, for 12 min at 30 °C, after preincubation at either 30 °C or 43 °C for 30 min. In each panel the first two lanes represent results from extracts preincubated at 30 °C, while the last two lanes represent extracts preincubated at 43 °C. The last lane on the right contains 7S RNA incubated without cell extract at 30 °C for 12 min. Quantitation of the experiment presented here demonstrates that at 0' (CP) the RNase E activity fell to 15%, compared with the nonheated extract, after thermal inactivation. In contrast, after the culture was subjected for an hour to chloramphenicol, the RNase E was at about the same level in the heated and nonheated extracts. The product of the RNase E cleavage (p5 rRNA) migrates slightly more slowly in the gel after the extract is preincubated at 43 °C. This is due to a conformational difference. Under these conditions, p5 often exhibits two conformers.

After the RNA is denatured with glyoxal, all the RNase E reaction products migrate as a single band

Carpousis et al. [16] presented evidence for a specific association between RNase E and polynucleotide phosphorylase. Antibodies raised against a fragment of *E. coli* RNase E cross-reacted with similar molecular weight proteins from a number of different bacteria [17].

The unexpected finding was recently made that *ams/rne* displays homology (mouse 32%; human 24%) with a gene coding for 2–5 A-dependent RNase, which is a uniquely regulated mediator of interferon action [6]. The speculations that the *ams/hmp1/rne* gene product might function to coordinate the overall biogenesis of RNA [4] and that it plays a general role in the bacterial cell may not be wide of the mark. Further studies should allow us to discover the reason for the unexpected observations.

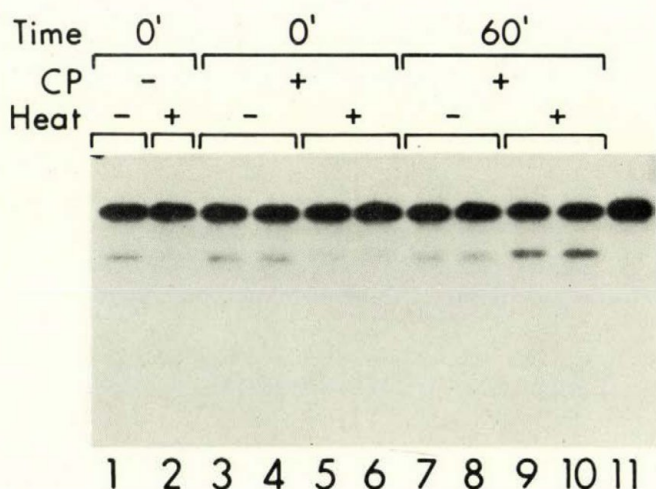


Fig. 4. Chloramphenicol treatment of strain N3438 increases the thermal stability of its RNase E^{ts}. N3438 was grown at 30 °C without chloramphenicol, with addition of CP just before (0') or 60 min before the cells were harvested. Extracts were prepared and heat-treated at 30 °C (lanes 1, 3, 4, 7, 8) or 43 °C (lanes 2, 5, 6, 9 and 10) for 30 min and assayed at 30 °C for 15 min with 7S RNA as substrate. Lane 11: 7S RNA incubated without cell extract

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COMPLEMENT C4, FACTOR B AND C3 POLYMORPHISM IN NORTH INDIA

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(Received November 4, 1994)

(Accepted November 18, 1994)

Allotype frequencies of complement C4, factor B and C3 proteins were determined in 192 healthy unrelated individuals from North India. The gene frequencies for the most common factor B and C3 alleles were close to the data already published. As expected, significant differences were observed in this first study of C4 allotype frequencies among the North Indian population in comparison to European Caucasians. According to historians, Hungarian Gypsies migrated from India. In this study we demonstrate that our data for some complement allele frequencies are in accordance with the opinion of historians.

The two cascades of the human complement system (classical and alternative pathways) consist of 20 proteins. The majority of these proteins are polymorphic. These polymorphisms may affect the antigenicity and/or functional activity of the respective component [1–4].

There are multiple data on frequency distributions of the complement protein allotypes in different ethnic groups [5], especially for the fourth component of the complement (C4), which has the highest number of allotypes. According to historians, Hungarian Gypsies migrated from India to Hungary through Spain. As the Gypsy population in Hungary still form a large, closed population, we were able to study the complement allotype frequencies of this ethnic group [6], and found significantly lower frequencies of the C4A*Q0 and C3*F alleles. Since there are only few publications on complement allotype frequencies and no studies are available on C4 allotype distribution in an Indian population, we investigated a limited number of individuals from the Northern part of India and compared them with Hungarian Gypsies.

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Materials and methods

EDTA-plasma samples were collected from 192 unrelated, healthy individuals (126 men and 66 women) belonging to New Delhi area. The samples were stored at -70°C and shipped to Hungary in dry ice.

C4 allotypes were determined according to Awdeh and Alper [7] using carboxypeptidase-B treatment as described by Sim and Cross [8]. To quantitate the ratio between C4A and C4B gene products the Coomassie-stained gels were evaluated by densitometric scanning [9].

Factor B allotypes were determined by cellulose-acetate electrophoresis, followed by immunofixation [10].

C3 allotypes were determined by the method of Teisberg [11].

For statistical analysis the STATGRAFICS software and for comparison of different ethnic groups, the Hardy-Weinberg test and chi-square test were used.

After determining the complement alleles, two sets of statistical analysis were done. In the first set, we compared the frequencies of the most common alleles in different ethnic groups using all the results. In the second set, we compared the allele frequencies of two different age groups: between the young group ($n=78$, mean age was 36.2 ± 11.7 years) and the elderly group ($n=51$, mean age was 64.0 ± 4.6 years) and compared them with results for Hungarian Caucasians.

Results and discussion

Results of the most common C4, C3 and factor B allele frequencies of the North Indian population are summarised in comparison with Hungarian Caucasians and Hungarian Gypsies in Table I (some very rarely occurring alleles are not listed in the table). These results show significant decrease in the frequencies of C4A*Q0 and C3*F alleles, and a significant increase in the frequency of Bf*F allele.

Until this time, only data on C3 and factor B allotype frequencies in the Asian population are available. Char and co-workers reported a gene frequency for C3*F allotype between 0.044 and 0.114 depending on caste subsets [12]. Similarly, Sahai and co-workers found 0.0172 for C3*F gene frequency in North India, from a random population around New Delhi [13]. The C3*F gene frequency reported for Hungarian Gypsies was 0.0079 [6], significantly lower than that obtained in this study (0.0445), though not significantly lower than that of the previously mentioned [14] results of the New Delhi population. As there are such large differences [12] even within the same caste depending on subsets, finding the population origin for Hungarian Gypsies would need a much wider study of the Indian population.

Only limited data are available for factor B allotype frequencies. According to Mauuff [5] the Bf*F gene frequency may vary practically from 0.00 to 1.00 according to ethnic groups. Miyano and co-workers [14] studied four Asian populations. They found a Bf*F gene frequency of 0.283 for the Filipino population as the closest to our results. Our previous results from the Hungarian Gypsy population showed frequency of 0.3069 for Bf*F, which does not differ significantly from that reported in this study for the North Indian population (0.2630).

Table I

Frequencies of the most common C4, C3 and factor B alleles in different ethnic groups

Allele	Hungarian Caucasians n=332		Hungarian Gypsies n=126		North Indians n=192	
	number	frequency	number	frequency	number	frequency
C4A*6	14	0.0210	12	0.0476	20	0.0521
C4A*4	35	0.0527	7	0.0277	46	0.1198*
C4A*3	457	0.6882	222	0.8809	273	0.7109
C4A*2	35	0.0527	6	0.0238	12	0.0312
C4A*Q0	115	0.1732	5	0.0198	31	0.0807*
C4B*3	22	0.0331	10	0.0396	6	0.0156
C4B*2	76	0.1144	7	0.0277	46	0.1198
C4B*1	476	0.7168	219	0.8690	301	0.7838
C4B*Q0	77	0.1159	16	0.0634	31	0.0807
C3*S	533	0.8027	250	0.9920	365	0.9505**
C3*F	111	0.1671	2	0.0079	17	0.0445**
Bf*S	534	0.8042	171	0.6785	274	0.7135**
Bf*F	123	0.1852	77	0.3069	101	0.2630**

* Significantly ($p < 0.001$) differs from Hungarian Caucasians as well as from Hungarian Gypsies** Significantly ($p < 0.001$) differs from Hungarian Caucasians

In terms of C4 allotype frequencies, we reported a significant decrease of C4A*Q0 allotype frequency in the Hungarian Gypsy population. In our present results, the frequency of the C4A*Q0 allotype was also significantly lower than that of the Caucasian population, but still significantly higher than that obtained for Gypsy population (0.1737, 0.0807 and 0.0217 for Hungarian Caucasians, North Indians and Hungarian Gypsies, respectively). Theoretically, this difference may be due to further selection during migration.

Comparing our previous finding on HLA frequencies of Hungarian, Gypsy, as well that of the limited data of Indian population, some HLA alleles also show significant differences [6]. The occurrence of HLA-A3 allele are 12.6% vs. 6.8% vs. 6.0%; for HLA-B5 are 7.0% vs. 14.2% vs. 13.7%; for HLA-DR2 12.0% vs. 19.5% vs. 25.6% (for Hungarian, Gypsy and Indian population, respectively).

As we have mentioned before, according to historians Hungarian Gypsies migrated from India. Comparing our previous and present findings, the most common complement allotype frequencies of Gypsies are closer to that of the North Indian population than to Caucasians. These data, however, do not provide direct genetic evidence for this hypothesis and need further confirmation.

Although the number of individuals tested in this study was limited, we made a comparison of C4 allotype frequencies in two age groups – young population under 50 years of age (n=78) vs. elderly population over 60 years of age (n=51). These data are summarized in Table II. For statistical analysis, the number of observed alleles in different age groups were compared within the same ethnic group. In addition, we compared the number of observed alleles of same age but different ethnic groups. Since the number of plasmas tested in the elderly group was small, the significant increase in the frequencies of the relatively rare C4A*4 and C4B*2 alleles may not represent real differences.

Table II

The frequencies of the most common C4 alleles in different age groups

Allele	Hungarian Caucasians				North Indians			
	young n=252		elderly n=485		young n=78		elderly n=51	
	number	frequency	number	frequency	number	frequency	number	frequency
C4A*6	9	0.0178	14	0.0144	9	0.0576 ⁺	4	0.0392
C4A*4	27	0.0535	47	0.0484	15	0.0961	19	0.1862 ⁺ *
C4A*3	371	0.7361	692	0.7134	115	0.7371	66	0.6470
C4A*2	34	0.0674	61	0.0628	4	0.0256	4	0.0392
C4A*Q0	63	0.1250	135	0.1391	13	0.0833	8	0.0784
C4B*2	61	0.1210	100	0.1030	15	0.0961	19	0.1862 [*]
C4B*1	355	0.7043	798	0.8226	125	0.8012	74	0.7254
C4B*Q0	81	0.1607	52	0.0536 [*]	12	0.0769 ⁺	9	0.0882

* Significant difference ($p < 0.01$) between the two age groups of the same population

⁺ Significant difference between the same age groups of different populations

Previously we reported a significant decrease of the C4B*Q0 allotype frequency in elderly population (0.1607 vs. 0.0539 for young and old population, respectively) [15]. This difference does not seem to exist in the presently studied population. There are several possibilities to explain this difference between the two ethnic groups. The first explanation is that the number of sera tested was too low. This may explain the differences in the frequency of a relatively rare allotype, such C4A*4 or C4B*2, but if there is a real tendency for decrease of a certain allele that is relatively often existing, we should have observed some significant changes.

The frequency of the C4B*Q0 allele among young population of North India was significantly lower (0.0780) than that of Caucasians (0.1607, $p = 9.8E-4$), and most probably arise from real ethnic differences between the two groups studied. The fact that there is no significant difference in the frequency of C4B*Q0 allele in different

age groups of North Indian population may be due to previous selections caused by different climate or nutrition.

Acknowledgement. This work was supported by Hungarian Research Found (OTKA 211) and Council of Scientific and Industrial Research, New Delhi.

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STUDY ON THE GLYCOPROTEIN NATURE OF CHICKEN IFNs BY CHEMICAL CLEAVAGE AND INHIBITION OF GLYCOSYLATION*

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(Received November 10, 1994)

(Accepted December 1, 1994)

The considerable molecular heterogeneity of chicken IFNs suggested the possible glycoprotein nature of these IFNs. The carbohydrate-specific oxidation and cleavage by neuraminidase indicated that fibroblast IFN may contain sugar moieties. Among the antimetabolites preventing glycosylation, monensin diminished IFN production. However, since it had the same effect on unglycosylated human leukocyte IFN, the drug very probably blocks intracellular transport. It rather proved that chicken IFNs are also secretory proteins. Tunicamycin diminished the formation of leukocyte IFN, but the decrease in titre of this IFN is due to the inhibition of protein synthesis. It is suggested that chicken fibroblast IFN is a glycoprotein, while leukocyte IFN lacks a sugar moiety. The IFN produced on induction with mitogen is closely related to leukocyte IFN, and therefore it seems that the chicken IFN system does not involve the conventional gamma type.

In humans and in mouse, a multiple gene family codes for different interferon-alpha (IFN- α) proteins and the molecular heterogeneity of IFN-beta and gamma coded for by single genes is due to the relative extents of glycosylation of the molecular components. We demonstrated that adenoviruses elicited IFN production in chick embryo fibroblast cells (CEF) and in leukocytes (FIFN and LIFN, respectively), and the production of acid-stable IFN (MIFN) has been demonstrated in a medium of leukocytes and spleen cells upon mitogen stimulation [1–3]. Similarly as for mammalian IFNs, our study of the physico-chemical characteristics of chicken IFNs has revealed considerable molecular heterogeneity [4]. Type-specific characteristics of avian IFNs, such as the glycoprotein nature or being the product of a multigens family, have not yet been described.

*This paper is dedicated to Professor Ilona Béládi, director emeritus of the Institute of Microbiology of Albert Szent-Györgyi University Medical School in Szeged, Hungary, by her pupils on the occasion of her 70th anniversary.

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As a possible explanation of the molecular heterogeneity, we presumed the glycoprotein nature of chicken IFNs. To obtain support for this notion, we investigated the carbohydrate-specific oxidation of IFNs by sodium periodate, enzymatic cleavage of sugar moieties, and the effects of antimetabolites preventing glycosylation of the IFN produced.

Materials and methods

Cell cultures and viruses. Primary CEF cells were cultured in Eagle's medium containing 5% calf serum. Peripheral blood mononuclear cells were isolated as described by Pusztai et al. [3] and maintained in Eagle's medium containing 5% calf serum. HEp-2 cells were grown as monolayers in the same medium. Human adenoviruses of type 5 (Ad5) and type 12 (Ad12) were propagated in HEp-2 cells and were purified as described elsewhere [5]. The Indiana serotype of vesicular stomatitis virus was used as challenge virus.

IFN induction and assay. IFN induction by Ad5 and Ad12 in CEF cells and leukocytes was performed as described by Béládi and Pusztai [1] and by Mucsi et al. [2], respectively. MIFN was induced by the method of Pusztai et al. [3]. In usual experiments, cells were infected at a multiplicity of 10^2 focus-forming units (FFU) of Ad12 per cell and 25 PFU Ad5 per cell, or were exposed to 10 µg/ml concanavalin A (Con A; Calbiochem) and incubated at 37 °C. IFN samples were harvested at 48 h postinfection.

IFN titration was carried out on secondary CEF cells by a micromethod described previously [5]. Purified IFNs were used in all experiments. The purification applied was described earlier [3]. The specific activities of LIFN, FIFN and MIFN were 2×10^5 , 10^4 and 10^3 IU/mg protein, respectively.

IFN induction in the presence of antimetabolites. The desired amounts of the drugs monensin and tunicamycin (Calbiochem) were added in a volume equivalent to one-tenth of that of the culture medium. Drugs were applied after 1 h of virus or mitogen adsorption and were present throughout the production period. In pulse-treatment experiments, the drug was added to the incubation medium at an appropriate time after induction and this medium was changed for fresh medium after the pulse period. Control cultures were treated in the same way except that the drug was omitted.

Evaluation of T antigen induction by Ad12. CEF were grown as monolayers on glass coverslips and 24 h after infection they were removed from the dish, washed in PBS, and fixed in acetone at 4 °C. The fixed cells were stained for T antigen by the indirect immuno-fluorescence technique described by Pusztai et al. [6].

Radioactive labelling. CEF were labelled with 3.7 MBq/ml ^{35}S -methionine in methionine-free medium for 60 min at different intervals after treatment with drugs. The acid-insoluble activity of TCA-extracted cells was determined.

Periodate and neuraminidase treatment. A 300 mM stock solution of sodium metaperiodate (Serva) was prepared in 0.1 M sodium acetate buffer at pH 4.5, and a 100 µg/ml solution of neuraminidase (*Clostridium perfringens*, Serva) in the same buffer at pH 5. Appropriate dilutions of purified IFNs were made in the pH 4.5 or 5 buffer and the reagents were added in one-tenth of the volume of the IFN samples. After a 1 h incubation at 37 °C, 5% ethylene glycol was added to the periodate and to the neuraminidase to stop the reaction.

Results

Oxidation of IFN by sodium periodate. Previous results have shown that, under appropriate conditions, sodium periodate oxidizes only the carbohydrate moiety of

glycoproteins [7]. Samples of purified LIFN, FIFN and MIFN were subjected to periodate oxidation at 37 °C for 1 h. The treatment had no effect on LIFN, but the mild oxidative cleavage by periodate decreased the titres of FIFN and MIFN in a concentration-dependent manner. On treatment with 30 mM periodate, the antiviral activity was eliminated completely (Table I).

Table I

Effects of periodate oxidation on antiviral activities of chicken IFNs

NaIO ₄ concentration (mM)	Antiviral titres as percentages of the control		
	LIFN	FIFN	MIFN
3	105	100	100
5	120	62	78
15	120	21	24
30	100	0	0

Effect of neuraminidase treatment of IFNs. Sialic acid is a common constituent of the sugar moieties of different IFNs. Corroboration of the carbohydrate nature of IFNs was sought in experiments where purified IFN samples were treated with neuraminidase. The enzyme treatment resulted in partial elimination of the antiviral activity of FIFN, the decrease at 10 µg/ml being 47%. LIFN and MIFN did not prove sensitive to neuraminidase treatment. In fact, even slight increases in the titres of these types were observed on treatment with lower concentrations of neuraminidase (Table II).

Table II

Effects of neuraminidase treatment on antiviral activities of chicken IFNs

Neuraminidase concentration (µg/ml)	Antiviral titres as percentages of the control		
	LIFN	FIFN	MIFN
2.5	105	80	105
5.0	120	65	120
10.0	100	53	85

Effects of monensin on IFN induction and T antigen formation. There is considerable evidence that monensin, a Na^+ ionophore is capable of interfering with glycosylation by blocking the processing of oligosaccharides. As an alternative approach to elucidation of the carbohydrate nature of chicken IFNs, we studied the effect of this antimetabolite on the induction by Ad12 of FIFN in CEF cells. Our results demonstrated that it has a reversible effect since treatment of the CEF cells before induction caused a negligible decrease in the antiviral titre (data not shown).

As illustrated in Fig. 1, when monensin was present in μM amounts during the induction, the FIFN yield was suppressed that the monensin concentration. At these concentrations, the drug did not influence the protein synthesis in treated cells. This was established by measuring the incorporation of ^{35}S -methionine into trichloroacetic acid-precipitable material.

It may also be assumed that monensin prevents the induction by exerting its effect on the inducer virus or on the virus-specific events preceding IFN formation. We therefore studied the T antigen formation in Ad12-infected CEF cells treated with 0.1–0.5 μM monensin. These cells, however, revealed normal T antigen morphology on examination with an immunofluorescence technique. There was no significant change in the number of T antigen-producing cells as compared with the infected, but untreated control. There was no difference between Ad5 and Ad12 inducers in respect of inhibition of IFN induction by monensin (Fig. 1).

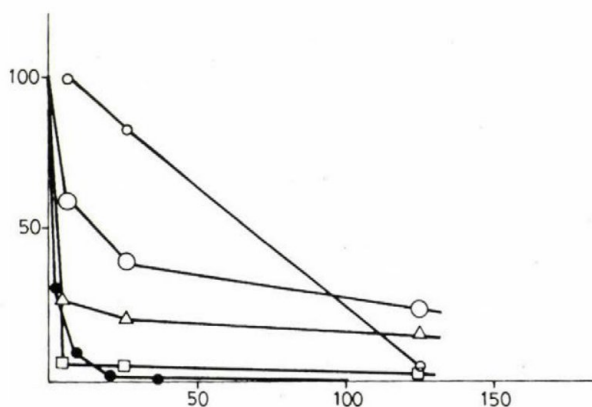


Fig. 1. Effects of monensin treatment on production of IFN by CEF cells induced by Ad5 or Ad12. Time of presence of the drug during the induction period, in hours: ○: –12–0 (pretreatment); □: 0–48; △: 12–48, ○: 24–48 in Ad5-infected cells, and ●: 0–48 in Ad12-infected cells. Abscissa: monensin concentration in μM ; ordinate: surviving IFN yield as percentage of the control

We also studied the effects of monensin on the LIFN production of chicken leukocytes on induction with Ad12, and on MIFN production on exposure to Con-A. A similar inhibitions was observed as in the case of FIFN (data not shown).

To examine the effect of monensin on the production of nonglycosylated human leukocyte IFN, we induced IFN by Sendai virus in human leukocytes treated with 0.01–3.5 μM monensin. We observed a dose-dependent inhibitory effect (Fig. 2).

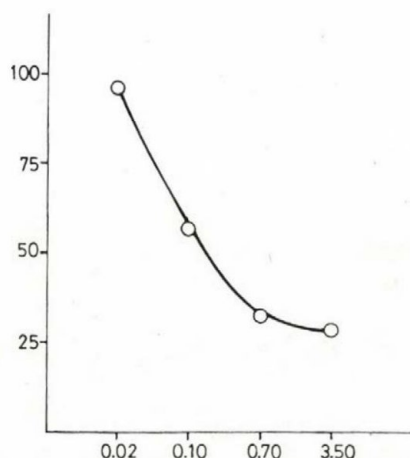


Fig. 2. Effect of monensin on production of IFN by human leukocytes induced by Sendai virus. The cells were treated during 0–20 h postinfection. Abscissa: Concentration of monensin in μM ; ordinate: IFN titre as percentage of control

In an attempt to pinpoint one specific event which is sensitive to monensin, we carried out pulse-treatment experiments. Infected cells were treated for various periods (before infection, or 0–48, 12–48 or 24–48 h postinfection). A specific event was not detected; instead, the effect of monensin prevailed throughout the induction process (Fig. 3).

IFN induction in the presence of tunicamycin. For an insight into the role of glycosylation, the effect of tunicamycin treatment on chicken IFN production was studied. In agreement with previous observations [8], the tunicamycin content of IFN samples interfered with their antiviral activity (interference with the assay system), and they were therefore dialysed overnight against Hanks solution before titration.

As illustrated in Fig. 4, there was significant difference between the sensitivities of IFN production by fibroblast cells and leukocytes. A decrease in FIFN yield could be observed at relatively low tunicamycin concentrations, whereas the production of LIFN diminished only at higher concentrations. Further, although the production of

FIFN was not totally suppressed even at high tunicamycin concentrations (500 µg/ml, 75–80% reduction) 100 µg/ml tunicamycin completely inhibited LIFN production.

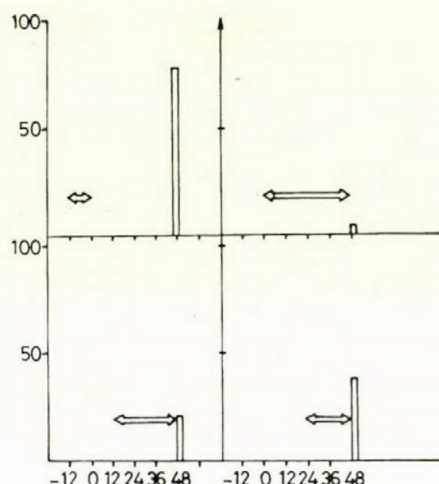


Fig. 3. Effect of pulse treatment with 0.25 µM monensin on yield of IFN production by CEF. The horizontal arrows show the time and length of the treatment. Abscissa: time in hours; ordinate: surviving yield of IFN as percentage of the control

To ascertain whether the inhibition is due to the overall shut-off of cellular protein synthesis, the rate of protein synthesis was measured at intervals via the incorporation of ^{35}S -methionine into trichloroacetic acid-precipitable material. As can be seen in Fig. 4, the protein synthesis of leukocytes can be inhibited more efficiently than that of CEF cells by tunicamycin. Thus, it seems that the apparent reduction in LIFN yield is due mainly to the general inhibition of protein synthesis. In similar experiments, the effect of tunicamycin on the formation of MIFN was analysed. The results closely resembled those obtained with LIFN (data not shown).

Discussion

The classification of IFNs was based on their antigenic properties and three major types are distinguished. It is widely accepted that beta and gamma IFNs are glycoproteins, whereas alpha IFNs are in general protein molecules not involving carbohydrates.

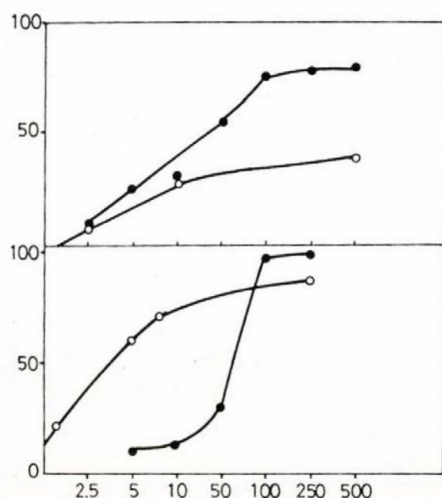


Fig. 4. Effects of tunicamycin on IFN production and protein synthesis in CEF cells and chicken leukocytes. Upper panel: CEF cells; lower panel: leukocytes. ●—● IFN production; ○—○ ^{35}S -methionine incorporation. Abscissa: tunicamycin concentration in $\mu\text{g/ml}$; ordinate: inhibition of IFN yield as percentage of control

Virus infection can elicit IFN production in fibroblast cells and leukocytes. In CEF and in chicken leukocytes, we used human adenoviruses as inducers, whereas in spleen cells, the mitogen Con-A was the inducer.

It is an intriguing problem to ascertain whether these IFNs are indeed the counterparts of the mammalian types. One possible approach is to analyse the carbohydrate nature of the avian IFN types, which was the aim of this study.

To elucidate the carbohydrate nature of chicken IFNs and unravel the molecular structure-biological activity relation, we used mild sodium periodate oxidation, which has been successfully applied to reduce the charge heterogeneity of human IFNs [9]. Our results showed that the biological activity of LIFN was not modified, whereas the antiviral effects of FIFN and MIFN were completely abolished by oxidation. The oxidation conditions chosen were sufficiently mild for the carbohydrate moieties to be preferentially oxidized, though the alteration of certain amino acid residues cannot be excluded.

We also made an attempt at enzymatic removal of carbohydrate groups. Since sialic acid is the most common carbohydrate constituent, we used neuraminidase for the treatment of chicken IFNs. This has been demonstrated to be efficient in the deglycosylation of human beta and gamma IFNs [9–12]. FIFN appeared to be the most susceptible to cleavage. We assume that the slight increases in titre at low enzyme concentrations may be due to the presence of an inhibitory factor which is preferentially destroyed by neuraminidase at the given concentration.

Further evidence was sought by studying the effects of antimetabolites preventing glycosylation. Monensin, a Na^+ ionophore, exerts its most profound effect on the trans cisternae of the Golgi apparatus. Among the functions of these, late processing such as terminal glycosylation is the most susceptible to inhibition by monensin. The reduced sialylation of immunoglobulin M and lymphoid cell surface glycoproteins was observed [13, 14]. Similarly, fibronectin was incompletely processed in the presence of the drug [15]. Monensin has been reported to decrease the galactosyl transferase activity of rat embryo fibroblasts [16]. It also blocked the conversion of viral membrane proteins to complex oligosaccharides in the case of herpes simplex virus and coronavirus [17, 18].

Monensin was not toxic for chick cells and did not interfere with Ad replication and T antigen formation. However, when it was present in μM amount during the induction of IFN, a concentration-dependent suppression of IFN yield was observed. Kinetic studies revealed that the suppressive effect was present throughout the induction process. A similar activity was demonstrated in the induction of FIFN, LIFN and MIFN.

In other studies, however, monensin was considered to act on the transport rather than on the processing per se [19, 20]. The incomplete processing is due to blockage of the transport in the Golgi apparatus. The inhibited transport of a great variety of secretory and membrane-associated molecules has been reported, including viral membrane proteins [21–24]. Coulombe and Skup [25] have published molecular genetic evidence that other secretory pathways independent of the signal peptide are not likely. If the production of human alpha IFN is inhibited by monensin, then, since glycosylation is not to be taken into account, it is only the secretion which may be impaired. Our results revealed that in fact, under similar conditions, the production of IFN induced by Sendai virus in human leukocytes could also be inhibited by monensin. It was therefore concluded that monensin rather blocks secretion and that, similarly to mammalian types, chicken IFNs are also secretory proteins.

Tunicamycin is a nucleoside-type antibiotic which inhibits the lipid carrier-dependent glycosylation of proteins. A survey of the relevant literature shows that the strategy of tunicamycin treatment varies among investigators, and fairly conflicting results have been published. For example the lack of the carbohydrate component reduced the intracellular solubility of IgM molecules and thus the secretion was impaired. In contrast, the secretion of unglycosylated IgA and thyroxine-binding protein was undisturbed [25–28]. The results are also at variance as regards the synthesis, assembly and maturation of glycoproteins of different enveloped viruses [29–32]. Nevertheless, tunicamycin treatment has been successfully used to reduce the molecular heterogeneity of human fibroblast IFN [33]. In this latter and other communications [34, 35], a considerable decrease in the yield of IFN upon tunicamycin treatment was reported. This is puzzling since the high specific activities achieved in prokaryotic systems which produce unglycosylated recombinant IFN have proved that the sugar moiety of IFN molecules is not indispensable for antiviral activity. The decrease in titre may be due to the modified folding of nascent IFN polypeptide whose glycosylation was impaired. This alteration in the folding may then result in a decreased stability or a low intrinsic specific activity.

We analysed the effect of tunicamycin on the production of chicken IFNs. Our results showed that the formation of FIFN is fairly sensitive to treatment with

tunicamycin, but it is not completely inhibited by the drug. IFN production in chicken leukocytes on induction with either Ad or mitogen proved to be more resistant to tunicamycin treatment. In contrast with CEF cells, a complete impairment of induction was observed in leukocytes at high concentrations. We assume, however, that this can be attributed to the overall shut-off of cellular protein synthesis.

These results lead us to conclude that FIFN is of a glycoprotein nature and is the counterparts of mammalian beta IFNs. LIFN and MIFN bear a close resemblance. Moreover, on the basis of their antigenic characteristics (to be published elsewhere), MIFN does not seem to be an independent type; we therefore assume that the chick IFN system does not involve a gamma IFN.

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307235

Acta Microbiologica et Immunologica Hungarica

VOLUME 42, NUMBER 4, 1995

(187)

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ACTA MICROBIOL. ET IMMUNOL. HUNG. 42 (4) 331-433 (1995) HU ISSN 1217-8950

ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN
ACADEMY OF SCIENCES

Acta Microbiologica et Immunologica Hungarica publishes reviews and original papers on microbiology and immunology in English

Acta Microbiologica et Immunologica Hungarica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1117 Budapest, Prielle K. u. 19-35

Manuscripts and editorial correspondence should be addressed to

Acta Microbiologica et Immunologica Hungarica
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Subscription price for Volume 42 (1995) in 4 issues US\$ 98, including normal postage, airmail delivery US\$ 20.00.

Acta Microbiologica et Immunologica Hungarica is abstracted/indexed in Abstracts of World Medicine, Biological Abstracts, Chemical Abstracts, Chemie-Information, Current Contents-Life Sciences, Excerpta Medica database (EMBASE), Index Medicus

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CONTENTS

Lactic Acid Production from Molasses by <i>Sporolactobacillus cellulosolvens</i> Kanwar, S. S., Tewari, H. K., Chadha, B. S., Punj, V., Sharma, V. K.	331
Antibodies to Spotted Fever Group Rickettsiae and <i>Coxiella burnetii</i> among Domestic Animals in Southern Croatia Punda-Polić, V., Poljak, S., Bubić, A., Bradarić, N., Klismanić-Nuber, Z.	339
Kinetic Characterization of Thyroid Peroxidase Enzyme and its Inhibition by Autoantibodies Kaczur, V., Vereb, Gy., Molnár, I., Krajczár, G., Balázs, Cs.	345
Seroepidemiologic Study of Anti-HAV IgG in Health-Care Workers Kassas, A. L., Mihály, I.	351
Comparison of Selected Species of <i>Bipolaris</i> , <i>Drechslera</i> and <i>Exserohilum</i> by Random Amplification of Polymorphic DNA Bakonyi, J., Pomázi, A., Fischl, G., Hornok, L.	355
Oxygen and Carbon Dioxide Requirements of <i>Helicobacter pylori</i> (A Note) Narikawa, S., Imai, N., Yamamoto, M., Suzuki, T., Yanagawa, A., Mizushima Y.	367
Microbial Counts and Characteristics of Streptovorticillium Strains Isolated from Soils in the Jordan Valley Mahmoud, J. I., Abussaud	373
Antibiotic Resistance of <i>Acinetobacter calcoaceticus</i> Strains Isolated from Patients Treated in Intensive Care Units Kiss, L., Leskő, T., Széll, M.	381
Is Seminal Fluid a Suitable Specimen for Detecting Chlamydial Infection in Men? Corradi, Gy., Konkoly Thege, M., Pánovics, J., Molnár, Gy., Bodó, Á., Frang, D.	389
Interaction Between <i>Bordetella pertussis</i> Vaccine and Chlorpromazine in Mouse Experiments Antmann, K., Anderlik, P., Ohár, A., Szeri, I., Bános, Zs.	395
A Higher Production of Platelet Activating Factor in Ex Vivo Heterologously Secondary Dengue-2 Virus Infections Yang, Kuender, D., Lee, Chuen-Shin, Shaio, Men-Fang	403
Radiodetoxified Endotoxin-Induced Tolerance. Effect on Endotoxin Lethality and Macrophage Arachidonic Acid Metabolism Bertók, L., Takáts, A., Hong Chen, Cook, J. A.	409
Serologically Verified Hantavirus Infections in Hungary Faludi, G., Ferenczi, E.	419
Role of Nitrogen Fixing Bacteria on the Phyllosphere of Wheat Seedlings Pati, B. R., Sengupta, S., Chandra, A. K.	427

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LACTIC ACID PRODUCTION FROM MOLASSES BY *SPOROLACTOBACILLUS CELLULOSOLVENS*

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(Received February 22, 1995)

(Accepted March 25, 1995)

Sporolactobacillus cellulosolvens (NCIMB 12173) isolated from an anaerobic digester and characterised biochemically is being reported for homofermentative lactic acid production from molasses in a batch culture. The effect of various process parameters on lactic acid production were optimized. A maximum lactic acid (24.2 g/l) and yield coefficient (0.79) was achieved using 3% (v/v) inoculum of 36 h old culture in molasses medium containing sugars (5%; w/v) supplemented with peptone (2.5 g/l) and $(\text{NH}_4)_2\text{SO}_4$ (7.5 g/l), pH 6.5 at 40 °C after 72 h of fermentation.

Lactic acid finds wider applications in pharmaceuticals, brewery, insecticides, biodegradable plastics, rubber, textiles, tanning and food industries as an acidulant and preservative [1, 2]. Lactic acid fermentation process is industrially carried out by homofermentative organisms mainly *Lactobacillus pentoses*, *L. casei*, *L. delbrueckii* and *L. bulgaricus*.

The substrates previously used for lactic acid production include molasses [1, 3], whey [4, 5], starch and corn sugar [6]. The nutritional requirements of lactobacilli are complex as they require supplementation of vitamins, amino acids, yeast extract, inorganic salts etc. [7] which render fermentation process uneconomical. *Sporolactobacillus cellulosolvens*, a spore former isolated from an anaerobic cattle waste digester is capable of fermenting a variety of substrates like sugars (glucose, galactose, mannose, fructose, sucrose, trehalose, arabinose, xylose and lactose), alcohols (manitol, sorbitol), starch as well as cellulose to lactic acid. Moreover, *S. cellulosolvens* is less fastidious in its nutritional requirements as compared to *Lactobacillus* spp. The present study envisages potentiality of *S. cellulosolvens* for the production of lactic acid from molasses by optimising various physiological parameters in a batch culture.

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Materials and methods

Microorganisms. *Sporolactobacillus cellulosolvens* NCIMB (National Collection of Industrial and Marine Bacteria) 12173, a micro-aerophilic spore former isolated from anaerobic digester was kindly provided by Dr. V. K. Sharma, Department of Microbiology, Punjab Agricultural University, Ludhiana, India. On nutrient agar, pinhead size colonies of *S. cellulosolvens* appear after 72 h incubation at 37 °C under micro-aerophilic conditions, while in oxygen deficient environment, bacterial colonies develop in 48 h.

Subculturing and maintenance of *S. cellulosolvens*. The strain was maintained in an agar based medium containing (g/l) sucrose (10.0), peptone (5.0), yeast extract (2.5), NH_4Cl (0.7), CaCO_3 (1.0), K_2HPO_4 (4.5), KH_2PO_4 (4.5), $(\text{NH}_4)_2\text{SO}_4$ (9.0), NaCl (0.9), MgSO_4 (0.9), CaCl_2 (0.9) and agar (20.0) and pH was adjusted to 7.0 using 0.5 N NaOH or H_2SO_4 . The slabs of medium were inoculated with *S. cellulosolvens* culture and incubated at 37 °C for 48 h. The stab cultures were later stored at 4 °C for further use.

Subculturing was carried out in broth i.e. maintenance medium without agar. The acid producing bacterial colony-forming units (CFU) were counted by employing standard pour plate method. The acid producing CFU were enumerated by flooding the plates with 0.01% (w/v) bromocresol purple which resulted in yellowish colour in and/or around the colonies.

Preparation of the inoculum. The inoculum of *S. cellulosolvens* for seeding the fermentation medium was prepared by aseptically transferring 1.0 ml of a 48 h culture to 100 ml of broth (containing molasses diluted to 18.8 g/l) in Erlenmeyer flask (250 ml capacity). The inoculated flasks were incubated at 37 °C for 24 h. The purity of the culture was determined by Gram staining before inoculating the fresh fermentation medium. In addition, the culture was also evaluated for purity by routine biochemical tests.

Preparation of fermentation medium. The fermentation medium contained (g/l): molasses (100.0), yeast extract (2.5), NH_4Cl (0.7), KH_2PO_4 (4.5), K_2HPO_4 (4.5), MgSO_4 (0.9), and CaCl_2 (0.9). The molasses was diluted to desired sugar concentration by addition of an appropriate volume of distilled water. The diluted molasses medium was thereafter percolated through a glass wool column to remove solid impurities. The pH of the molasses medium was adjusted to 7.0 with 0.5 N NaOH. One hundred ml of this medium was distributed in each of the Erlenmeyer flask (250 ml capacity) and was autoclaved at 1.1 bar pressure for 20 min.

Optimization of physiological parameters for fermentation. The effect of process parameters such as: age of inoculum, level of inoculum, initial sugar (diluted molasses) concentration, pH of the fermentation medium, organic (peptone) or inorganic (ammonium sulphate) nitrogen source(s) and fermentation temperature on the lactic acid production by *S. cellulosolvens* in batch culture was evaluated. The fermentation was regularly monitored by quantitative analysis of lactic acid concentration [8], total reducing sugars, and residual sugars [9] in the fermented mash. Appropriate uninoculated controls were included in each of the experiments for the estimation of initial total sugars content and the presence of traces of lactic acid (if any) in the uninoculated medium.

Statistical analysis. All the experiments were performed in triplicates. The mean standard error was calculated and statistical significance was evaluated at 95% confidence limit by Student's *t*-test.

Results

The total sugar content in the molasses was estimated to be 49.8% which had 27.5% reducing and 22.3% non-reducing sugar. *S. cellulosolvens* was found to have maximum specific growth rate ($\mu=0.298/\text{h}$) and a generation time of 2.04 h. A lag phase of approximately 6 h was observed and thereafter, a gradual increase in the

viable cell count from 6.0×10^7 to 1.85×10^{10} CFU/ml was recorded after 48 h of incubation. The lactic acid production was observed to be maximum (9.6 g/l) after 72 h of fermentation corresponding to 72.3% sugars utilization (Fig. 1).

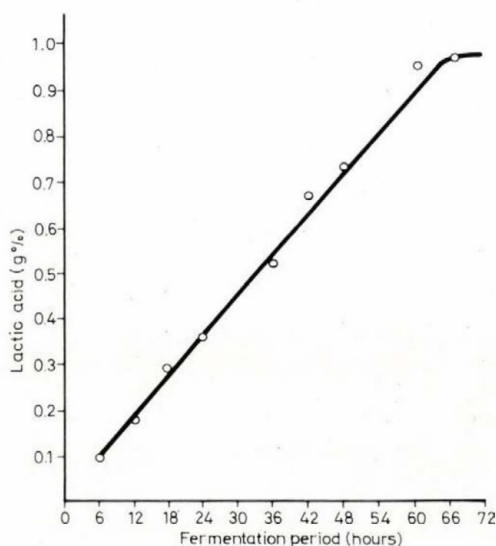


Fig. 1. Lactic acid production by *S. cellulosolvens*

Effect of inoculum age. *S. cellulosolvens* culture grown for 12, 26, 36 and 54 h representing lag, early log, mid-log and stationary phase of growth of the organism, respectively, was used as inoculum. The results (Table I) showed that the maximum decrease in pH with relatively higher lactic acid production (14.7 g/l) and a product yield ($p/s=0.56$) was obtained in medium inoculated with 36 h old culture.

Effect of inoculum level. An increase in the level of inoculum from 1.0% to 3.0% (v/v) resulted in considerable increase in the lactic acid production from 16.1 to 18.1 g/l after 72 h of fermentation with simultaneous increase in sugar uptake (Table II). However, any further increase in inoculum level up to 15.0% resulted in only slight increase to 18.8 g/l lactic acid.

Effect of initial sugars (molasses) concentration. An initial sugar concentration of 5.0% (w/v) was found to be optimum for lactic acid production, where the lactic acid (18.6 g/l) was recorded with 50% substrate utilization representing a product yield of 0.74 (Table III).

Table I

Effect of age of inoculum on lactic acid fermentation by S. cellulosolvens

Age of culture period, h	pH*	Lactic acid g/l	% Sugars utilized	Y' (p/s)**
Initial	7.0	—	—	—
12	5.6	7.5 (±0.05)	42.1	0.35
26	4.2	8.9 (±0.06)	45.1	0.39
36	3.6	14.7 (±0.12)	52.5	0.56
54	3.6	14.5 (±0.09)	53.4	0.54

Working volume, 100 ml.; Sugar conc., 49.2 g/l; temperature, 37 °C; pH, 7.0; inoculum level, 1.0% (v/v); fermentation time, 72 h

* Final pH

** Lactic acid yield = $\frac{\text{lactic acid produced, g}}{\text{substrate utilized, g}}$

Figures given in parentheses indicate mean standard error (MSE)

Table II

Effect of inoculum level on lactic acid fermentation by S. cellulosolvens

Inoculum %, v/v	Lactic acid g/l(MSE)	% Sugars utilized	Y' (p/s)
1.0	16.1 (±0.03)	47.8	0.67
3.0	18.1 (±0.05)	49.2	0.73
5.0	18.5 (±0.06)	49.5	0.73
7.0	18.5 (±0.12)	50.0	0.74
9.0	18.5 (±0.09)	50.6	0.74
11.0	18.7 (±0.11)	50.8	0.75
13.0	18.8 (±0.08)	50.8	0.74
15.0	18.8 (±0.12)	50.9	0.74

Working volume, 100 ml; initial sugar conc., 49.2 g/l; temperature, 37 °C; pH, 7.0; inoculum level, 1.0% (v/v) 36 h old culture; fermentation time, 72 h

Table III

Effect of sugar concentration on lactic acid fermentation by S. cellulosolvens

Sugar conc. g/l	Lactic acid g/l (MSE)	% Sugars utilized	Y'=(p/s)
9.8	7.6 (± 0.07)	78.21	0.97
29.5	14.4 (± 0.09)	62.02	0.77
49.2	18.6 (± 0.09)	50.00	0.74
68.9	18.6 (± 0.11)	39.48	0.67
88.6	15.8 (± 0.05)	31.36	0.56
108.3	15.8 (± 0.10)	26.47	0.54

Working volume, 100 ml; temperature, 37 °C; pH, 7.0; inoculum, 3.0% (v/v) 36 h old culture; fermentation period, 72 h

Effect of H⁺-ion concentration. The lower initial pH of the fermentation medium appears to have a negative effect on the lactic acid production. It was observed that by varying the pH of the molasses medium from 5.0 to 6.5, the lactic acid concentration in the fermented mash increased from 8.2 to 18.6 g/l (Table IV). However, the fermentation carried out at an initial pH>6.5 prompted an increased sugars utilization but with a poor lactic acid yield.

Table IV

Effect of pH on lactic acid fermentation by S. cellulosolvens

Initial pH	Final pH	Lactic acid g/l (MSE)	% Sugar utilized	Y'=(p/s)
5.0	4.39	8.2 (± 0.09)	21.4	0.77
5.5	4.43	9.5 (± 0.12)	25.2	0.75
6.0	3.91	15.5 (± 0.17)	41.2	0.75
6.5	3.61	18.6 (± 0.13)	48.4	0.77
7.0	3.64	16.8 (± 0.14)	49.2	0.68
7.5	3.81	16.9 (± 0.09)	52.8	0.64
8.5	4.55	16.0 (± 0.10)	51.2	0.63

Working volume, 100 ml; temperature, 37 °C; inoculum, 3.0% (v/v) 36 h old culture; initial sugar, 49.2 g/l; fermentation time, 72 h

Table V

Effect of nitrogen sources on lactic acid fermentation by S. cellulosolvens

Nitrogen* source(s)	Lactic acid g/l (MSE)	% Sugar utilized	Y'=(p/s)
KNO ₃	11.0 (±0.12)	27.2	0.81
NaNO ₃	11.8 (±0.13)	51.7	0.74
(NH ₄) ₂ SO ₄	20.7 (±0.09)	58.4	0.71
NH ₄ Cl	11.8 (±0.13)	54.1	0.69
Urea	11.2 (±0.16)	20.8	0.95
Peptone	20.8 (±0.09)	47.0	0.89

Working volume, 100 ml; temperature, 37 °C; pH, 6.5; inoculum, 3.0% (v/v) 36 h old culture; initial sugar, 49.2 g/l; fermentation period, 72 h

* Nitrogen sources added to 0.2% nitrogen content

Effect of nitrogen source(s) on lactic acid production. The supplementation of fermentation medium with peptone resulted in maximum lactic acid production (20.8 g/l) and a product yield of 0.89. Moreover, it was found that inorganic nitrogen source (NH₄)₂SO₄ was equally suitable for supporting fermentation whereby, a lactic acid concentration (20.7 g/l) and a product yield 0.71 was achieved after 72 h of fermentation (Table V). The effect of simultaneous presence of (NH₄)₂SO₄ and peptone in different ratio(s) was also evaluated. It was observed that maximum production of lactic acid (22.7 g/l), 57% sugar utilization and product yield of 0.79 could be achieved when peptone and (NH₄)₂SO₄ were supplemented to the fermentation medium in a ratio of 1:3 (Table VI).

Table VI

Effect of organic and inorganic nitrogen source on lactic acid fermentation by S. cellulosolvens

Nitrogen source		Lactic acid	% Sugars utilized	Y'=(p/s)
(NH ₄) ₂ SO ₄	Peptone	g/l (MSE)		
% w/v	% w/v			
0.75	0.25	22.7 (±0.21)	57.4	0.79
0.50	0.50	19.4 (±0.13)	49.2	0.79
0.25	0.75	20.9 (±0.14)	52.9	0.79

Working volume, 100 ml; temperature, 37 °C; pH, 6.5; inoculum, 3.0% (v/v) 36 h old culture; initial sugar, 49.2 g/l; fermentation period, 72 h

Table VII

Effect of temperature on lactic acid fermentation by S. cellulosolvens

Fermentation temperature, °C	Lactic acid g/l (MSE)	% Sugar utilized	Y'=(p/s)
25	12.2 (±0.06)	46.5	0.53
30	16.9 (±0.10)	52.1	0.65
35	19.3 (±0.08)	57.8	0.67
40	24.2 (±0.14)	61.0	0.79
45	21.0 (±0.18)	59.0	0.71

Working volume, 100 ml; pH, 6.5; inoculum, 3.0% (v/v) 36 h old culture; initial sugar, 49.2 g/l; fermentation period, 72 h

Effect of fermentation temperature. An increase in fermentation temperature from 25 °C to 40 °C resulted in a significant ($p < 0.5$) increase in lactic acid production and sugar utilization (Table VII). A maximum lactic acid (24.2 g/l) was obtained at a fermentation temperature of 40 °C at 72 h post-inoculation. The lactic acid fermentation at a temperature of 45 °C significantly decreased the lactic acid production.

Discussion

S. cellulosolvens, a sporeforming homofermentative Gram-positive bacterium is capable of utilizing a wide variety of sugars and can efficiently ferment molasses to lactic acid. The molasses fermentation was found to be optimum by using mid-log phase inoculum (36 h old culture). The higher cell count in mid-log phase (1.14×10^{10} /ml) reduced the amount of inoculum to be used to 3.0% (v/v) in contrast to 10.0% (v/v) used for fermenting whey to lactic acid as reported earlier by our laboratory [5] and thus is effective for process economics.

S. cellulosolvens is affected at increased molasses concentration which may be attributed to lower osmotolerance of the organism as well as the presence of certain toxic substances such as waxes, plant pigments, sterols etc. which generally exist as contaminants in molasses [10]. A pH of 6.5 was found suitable for lactic acid production indicating homolactic fermentation as Gomez and Coronado [11] found that at a lower pH the principal end-product was always ethanol while at higher pH (approaching neutrality) the end-product was lactic acid. Moreover, pH affects the availability of metal ions in the medium [11, 12].

It appears that the supplementation of peptone as a nitrogen source in the medium stimulated the growth of *S. cellulosolvens* instead of promoting lactic acid production. Since, the variation in the ratio of peptone: $(\text{NH}_4)_2\text{SO}_4$ from 1:1 to 3:1 increased the utilization of sugars in the molasses medium significantly ($p < 0.05$) without a marked

increase in the lactic acid. In contrast, $(\text{NH}_4)_2\text{SO}_4$ in the medium favoured lactic acid production as it is more readily metabolisable than peptone. Moreover, it also possesses a good buffering capacity [12].

The temperature seems to have pronounced effect on the rate of lactic acid production by lactobacilli and the product output roughly doubles for each 10 °C rise in temperature over a range of 25 to 40 °C. The same appears to be true for lactic acid fermentation of molasses by *S. cellulosolvens*. Most of the lactobacilli have a temperature range of 40 °C to 50 °C for optimal production of lactic acid. Different lactate dehydrogenases exert maximum activity at different temperatures [13]. A relatively higher production of lactic acid at 40 °C perhaps indicates the presence of a lactate-dehydrogenase isoenzyme system in *S. cellulosolvens* which possesses an optimal activity at 40 °C.

From the foregoing discussion it may be suggested that for an efficient lactic acid production by *S. cellulosolvens* in batch fermentation of molasses medium, the process must be carried out with an initial sugar concentration of 5.0% (w/v), pH of the medium adjusted to 6.5 at 40 °C. The nitrogen sources i.e. peptone (0.25%; w/v) and $(\text{NH}_4)_2\text{SO}_4$ (0.75%; w/v) should be added to the medium. The end product yield can be further improved by the use of immobilized *S. cellulosolvens* cells or by employing continuous mode of fermentation.

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ANTIBODIES TO SPOTTED FEVER GROUP RICKETTSIAE AND *COXIELLA BURNETII* AMONG DOMESTIC ANIMALS IN SOUTHERN CROATIA

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(Received June 17, 1994)

(Accepted August 17, 1994)

The sera of dogs, goats, sheep and cattle (total 153) from the Kastelas' bay area (middle part of the eastern coast of the Adriatic Sea) were tested for antibodies against *Rickettsia conorii* and *Coxiella burnetii* by a complement fixation test. The overall percentages of positive sera among the tested animals were 23.9% for *R. conorii* and 16.4% for *C. burnetii*. The results show that animals in this area are clearly being exposed to spotted fever group rickettsiae as well as to *C. burnetii*. For comparison, six sheep sera from the flock, living in known Q fever focus in hinterland area and linked with outbreak of Q fever among owners were tested. Antibodies to *C. burnetii* but not for *R. conorii* were present in all six sera.

In many countries rickettsial infections appear frequently and, under certain circumstances, can cause a serious public health problem. Since the natural cycle of infection involves animals, they may serve for evaluation of the prevalence of rickettsia infection within specific area [1, 2].

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There is no contemporary report in Croatia on prevalence of antibodies against rickettsia in domestic animals. Previous serologic survey carried out in Croatia demonstrated only antibodies against *C. burnetii* (the agent of Q fever) in domestic animals [3–5]. No studies about the circulation of rickettsia in the middle part of Southern Croatia (eastern coast of Adriatic Sea) have yet been done.

The first well-documented cases of the Mediterranean spotted fever (MSF) caused by *R. conorii* in Croatia were reported among inhabitants and tourists in Kastelas in 1982 [6]. Since then, a high frequency of human cases of MSF in this region has been observed and the area of Kastelas' bay is considered as a *R. conorii* focus. The dog which is the primary host of *R. conorii*, and its most common vector, the brown dog tick *Rhipicephalus sanguineus* [7] are common in the studied area as well as in the whole southern Croatia [8]. So far only sporadic cases of Q fever, caused by *C. burnetii* among the population of Kastelas have been recognized [9].

The purpose of this work was to investigate the prevalence of antibodies against spotted fever group rickettsia (*R. conorii*) and *C. burnetii* among domestic animals living in the studied area. The limited amount of data on the activity of rickettsia in Croatia suggested the usefulness of such a study.

Materials and methods

Study area. The Kastelas' bay region is located in the middle part of Southern Croatia (middle part of the eastern Adriatic coast). There are seven semi-rural communities scattered around the bay (Fig. 1).

Serum samples. Serum specimens from 153 domestic animals from the region of Kastelas' bay were studied in this survey: from 51 dog, 38 from goat, 11 from cattle and 53 from sheep, collected from June 1991 to March 1992. In addition, blood samples were drawn from the flock (6 sheep) in the village near Sinj (in hinterland area; Fig. 1). These sheep belonged to the household where at that time (February 1992) among owners a clinically and serologically confirmed family outbreak of Q fever had been observed. The region of Sinj is known as a Q fever focus [10].

Animal sera were collected from the jugular vein (cattle, goats, and sheep) and from the cephalic vein in dogs. The sera were stored at -20°C until tested.

All sera were analysed by the complement fixing (CF) test. The CF test was performed according to the standard procedure with 2 units of antigen [11] using as antigens *R. conorii* (strain Simko), and *C. burnetii* (strain Nine Mile phase II) prepared in yolk sacs of embryonated chicken eggs (Institute of Public Health, Zagreb, Croatia). Serum titres of $\geq 1:4$ were considered evidence of prior infection by rickettsia. The significance and specificity of this titre was substantiated in the earlier investigations [12, 13]. Each test was performed with antigen, serum, complement, and haemolytic system controls [11].

Results

The proportions of domestic animals sera giving a positive reaction against *R. conorii* and *C. burnetii* are shown in Table I. Overall, 38 (23.9%) sera had CF antibodies to *R. conorii* and 26 (16.4%) to *C. burnetii*. The highest frequency of antibodies against *R. conorii* was found in dog sera (52.9%). Three (7.9%) of 38 goat sera, three (27.3%)

of 11 bovine sera, and five (9.4%) of 53 sheep sera showed positive reaction for *R. conorii*. Antibodies against *C. burnetii* were revealed in 6 canine (11.8%), six caprine (15.8%), two bovine (18.2%) and six (11.3%) ovine sera. All six sheep sera from the village near Sinj (surveyed in connection with the epidemic of Q fever) had antibodies for *C. burnetii*.



Fig. 1. Map of Croatia, showing the localities (Kastela's bay and Sinj) where serum samples were collected

Table II shows titres and geometrical mean titres (GMT) of positive sera. The highest frequency of *R. conorii* positive sera was found in dogs, higher than in cattle, sheep and goats, but the overall level of antibody titres was in general low. CF antibody titres to *R. conorii* higher than 1:32 were detected only in canine sera (GMT=10.6). In all tested animal sera from area of Kastela titers of antibodies to *C. burnetii* were low.

On the other hand, the rate of CF antibodies for *C. burnetii* in sheep from the flock whose owners had family epidemic of Q fever was high (100%) with the titre level ranging up to 1:128, which was indicative of recent infection. Geometrical mean titre was significantly lower in sheep sera from Kastelas region (GMT=5.6) than that observed in flock linked with human cases of Q fever in the vicinity of Sinj ($t=14.7$, $p<0.01$) [14].

Table I

Frequency of complement fixing antibodies to R. conorii and C. burnetii in sera from animals in the middle part of eastern Adriatic coast

Sera	No. of sera tested	Sera positive to			
		<i>R. conorii</i>		<i>C. burnetii</i>	
		No.	%	No.	%
Dog	51	27	52.9	6	11.8
Goat	38	3	7.9	6	15.8
Cattle	11	3	27.3	2	18.2
Sheep	53	5	9.4	6	11.3
Sheep*	6	0	0	6	100.0
Total	159	38	23.9	26	16.4

* Sheep connected with family outbreak of Q fever in the hinterland area of Sinj

Discussion

Several serological tests have been developed to detect antibodies reactive with rickettsiae. We used CF test considering it satisfactory for a seroepizootiological survey [12, 13].

In our study 52.9% of the dogs had antibodies to *R. conorii*. Data from other authors from the Mediterranean area showed a higher prevalence of canine antibodies to the agent tested by indirect fluorescent antibody test. Thus, high percentages, more than 80% in several areas, were obtained in canine population from western Sicily [15], and nearly 80% seroprevalence was reported in the south of France [16]. The prevalence of antibodies in dogs ranged from 58.6% in central Spain [17] to 93% in Salamanca [18]. The discrepancies between our and other authors' data may reflect the differences in the methods used, because CF is less sensitive than the indirect fluorescent antibody test [19]. Our finding of antibodies to *R. conorii* in the other domestic animals tested (goats,

cattle and sheep) is in an agreement with the finding of Herrero-Herrero et al. [18] in Spain. The diversity of animals with CF antibodies to *R. conorii* suggest that other vertebrates besides dogs can play a role in the epizootiological chain of spotted fever group rickettsiae.

Table II

Distribution and geometrical mean of antibody titers against R. conorii and C. burnetii in positive animal sera

Sera	<i>R. conorii</i>						<i>C. burnetii</i>						
	4	8	16	32	64	GMT	4	8	16	32	64	128	GMT
Dog	9	6	6	4	2	10.6		6					8.0
Goat	1		2			10.1	1	3		2			11.3
Cattle	1	1	1			8.0	1	1					5.7
Sheep	2	2	1			6.9	4	1	1				5.7
Sheep*							1		1	1	1	2	35.9

GMT= Reciprocal values of geometrical mean titres

* Sheep from the flock linked with epidemic of Q fever

Results of our study also show that CF antibodies to *C. burnetii* were found in all animals tested. This is supportive to the notion that *C. burnetii*, agent of Q fever, is one of the most common rickettsiae in Croatia, both in humans and domestic animals. Prevalence of antibodies to *C. burnetii* in this study is similar to that recorded in animals (sheep, goat and cattle) on islands and Croatian littoral [3-5]. For the first time in Croatia we have demonstrated antibodies to *C. burnetii* in canine sera. Our data pointed out that all sheep connected with outbreak of Q fever (in hinterland area) had antibodies with high titers (GMT=35.9) to *C. burnetii* indicative of a recent infection, and suggests it to be the main cause of the outbreak among household members. In sheep sera from Kastelas region the frequency of positive sera (11.3%) and the antibody titres (GMT=5.7) were significantly lower which points to little activity of the agent among sheep at that time in the surveyed area. Similar to this Vesenjok-Hirjan and Hrabar [4] found in the course of Q fever epidemic in northern Croatia that 93% of examined sheep from affected area had CF antibodies to *C. burnetii*. Obtained titres of CF antibodies were significantly higher than titres among sheep in areas where no recent infection in humans occurred [4].

Based on the serologic evidence the present study allows the conclusion that in the whole surveyed territory there are enzootic foci of *C. burnetii* and spotted fever group rickettsiae. The finding points to the need for further studies on the epizootiology of the rickettsiae in Croatia, with particular emphasis on the isolation of rickettsia.

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KINETIC CHARACTERIZATION OF THYROID PEROXIDASE ENZYME AND ITS INHIBITION BY AUTOANTIBODIES

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(Received November 2, 1994)

(Accepted December 1, 1994)

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A guaiacol method was developed to measure thyroid peroxidase (TPO) activity and the inhibitory effect of anti-TPO antibodies using purified porcine TPO. The TPO preparation was characterized kinetically and identified by western-blotting technique. The K_M for guaiacol determined in vitro was 5.6×10^{-4} M. Investigating the effect of IgG's from 23 hypothyroid patients on TPO activity inhibition was detected in 15 cases with the guaiacol method. It was found that anti-TPO antibodies exerted a competitive inhibition TPO activity with respect to the substrate guaiacol. The inhibition is carried by IgG(ab')₂ fragment. We wanted to gain a method which is interpreted immunologically and can be well employed in the clinical practice.

Thyroid peroxidase (TPO) has been recognized as a major thyroid microsomal antigen (M/TPO) [1, 2] involved into the autoimmune thyroiditis [3, 4]. TPO is an integral part of thyroid membrane which plays a key role in the iodination of tyrosyl residues in thyroglobulin and other proteins [5–7]. It is a glycoprotein [9, 10] of which SH or S-S [5] groups are important in the enzyme activity and sensitive to reductive agents [11]. Two methods are widely accepted for determination of TPO based on oxidation of iodide or guaiacol, respectively [5, 12], on the enzyme there are two binding sites of active centre for the second substrate [5, 12, 13]. Deriving from the integral membrane protein nature of the TPO, it is difficult to get an antigen with proper purity and activity. The polyclonal anti-TPO antibodies from AITD, which binding to TPO, part of antibodies are able to inhibit the activity of enzyme. Since the TPO and the inhibition of the TPO autoantibodies have not been described kinetically so far, our present work was aimed to perform this characterization. On the other hand, we wanted

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to gain a method (using the antibodies which have been screened by the ELISA technique and then purified by different procedures) which is interpreted immunologically and can be well employed in the clinical practice.

Materials and methods

Preparation of porcine thyroid-peroxidase (TPO) was performed essentially according to Neary et al. [5]. Porcine thyroid glands were ground and washed four times by suspending in 1.15 M KCl/ 10^{-4} M KI and centrifuging (5 000 g, 40 min). Then the tissue was homogenized with 0.25 M saccharose / 10^{-4} M KI, filtered through cheese-clothes and the filtrate was centrifuged (5 000 g, 20 min). From the supernatant the microsome fraction was pelleted at 50 000 g for 180 min and washed successively with solutions of 1 M NaCl / 10 mM Tris-HCl / 10^{-4} M KI, pH 7.4 and then with 10^{-4} M KI. The washed microsome fraction was treated with chymotrypsin-free, DPCC-treated trypsin (35 U/mg protein) (Merck, Germany) at 37 °C for 1 h and the reaction was terminated with soybean trypsin inhibitor (2 mg/mg trypsin). The supernatant obtained at 105 000 g for 1 h was applied to an 1.6×22 cm anion-exchange column (DE-52, Whatman, Germany), equilibrated with 25 mM KCl / 10^{-4} M KI / 15 mM Tris-HCl, pH 7.4. The TPO activity was eluted by applying a linear KCl gradient of 25–225 mM KCl in 10^{-4} M KI / 15 mM Tris-HCl, pH 7.4. The pooled fractions were concentrated on a PM-30 ultrafiltration membrane (Amicon, Switzerland), applied to a 1.6×85 cm Sephacryl S-200 (Pharmacia, Sweden) column equilibrated with 50 mM K-phosphate / 10^{-4} M KI, pH 7.2 and eluted with the same buffer. The active fractions were concentrated as before and stored frozen at -70 °C.

Identification of purified TPO fragments. The purified porcine TPO was controlled by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [14] and by Western-blotting technique [15].

TPO activity measurement. Standard determination of TPO activity was performed in a cuvette with 1.0 cm light path containing 33 mM guaiacol (Fluka, Germany) in 1 ml 0.1 M phosphate buffer, pH 7.4, and 0.3 mM hydrogen-peroxide (Merck, Germany). The reaction was initiated by adding H_2O_2 . Extinction was read at 470 nm per 15 sec intervals at least for 1 min using a spectrophotometer. The rate of reaction was calculated using an extinction coefficient of $5.75\text{ cm}^{-1}\text{ mM}^{-1}$ for oxidized guaiacol [5]. One unit (U) was defined as the amount of enzyme which transformed 1 μmol guaiacol per min. For kinetic measurements the guaiacol concentrations varied between 0.5 mM and 48 mM, and the H_2O_2 concentrations between 0.3 and 0.85 mM.

Isolation of IgG isotype of anti-TPO antibodies. IgG was obtained from sera of patients or healthy controls by precipitation with polyethylene-glycol (PEG) (mol wt:6000, Fluka, Germany) [16]. The IgG isotype anti-TPO antibodies were prepared by affinity chromatography on a Protein G-Sepharose (Pierce, USA) column. The eluted antibodies were desalted by gel filtration. To obtain IgG fragments the IgG solution was treated by pepsin (used by the description given) and the resulting IgG Fc and IgG F(ab')₂ fragments were separated using Protein A-Sepharose column. Elution of IgG (in one peak) was followed at 280 nm.

Inhibition of TPO activity by antibodies. In general 3.4×10^{-6} M, in the kinetic measurements 1.8×10^{-6} M to 10.5×10^{-6} M IgG (mol wt:156 000) separated with PEG was employed, and 3.7 mg/ml IgG F(ab')₂ was used. The solution containing anti-TPO antibodies with TPO shaking at 23 °C or 37 °C. Controls with IgG of healthy persons were also incubated in the same way. The absorbancy of IgG was corrected in every measurement.

Results

1. The purified TPO being controlled by SDS-PAGE and Western-blotting technique, the antigen showing immunoreactivity was at 105 and 110 kD, at non-reducing conditions (data not shown).

2. The specific activity of final porcine TPO preparation was 14.18 U/mg protein determined with guaiacol method, this was similar to that reported Neary et al. [5].

3. The K_M for guaiacol determined in vitro according to Lineweaver-Burk was 5.6×10^{-4} M while K_M values of 10^{-4} M, 3×10^{-6} M and 6×10^{-3} M were found for protein iodination [9], Glu-Tyr-Glu iodination [10], or iodide-oxidation [17], respectively.

4. To characterize the type of inhibition we have applied various substrate and inhibitor concentrations and found a purely competitive inhibition with guaiacol substrate in the classic determination [18], according to Lineweaver-Burk (Fig. 1). The inhibitor coefficient was determined by Dixon's diagram as 2.01×10^{-5} M for IgG (Fig. 2), i.e. representing $1/v$ as a function of $[I]$ and using different $[s]$, abscissa of the common crucial point of the lines gives directly the inhibitor coefficient.

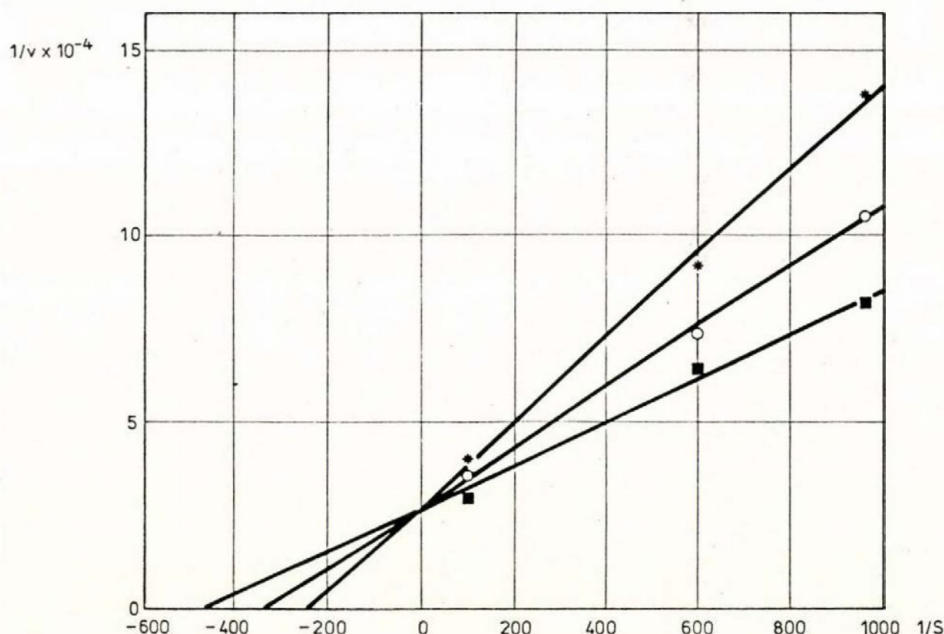


Fig. 1. Kinetic characterization of inhibition on TPO enzyme by IgG isotype anti-TPO antibodies according to Lineweaver-Burk. To characterize type of inhibition, we applied various substrate and inhibitor concentrations (given in the Methods). ○, * reaction mixture with IgG isotype anti-TPO antibodies; ■ without antibodies; (v is calculated as $\text{nmol oxidized guaiacol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$)

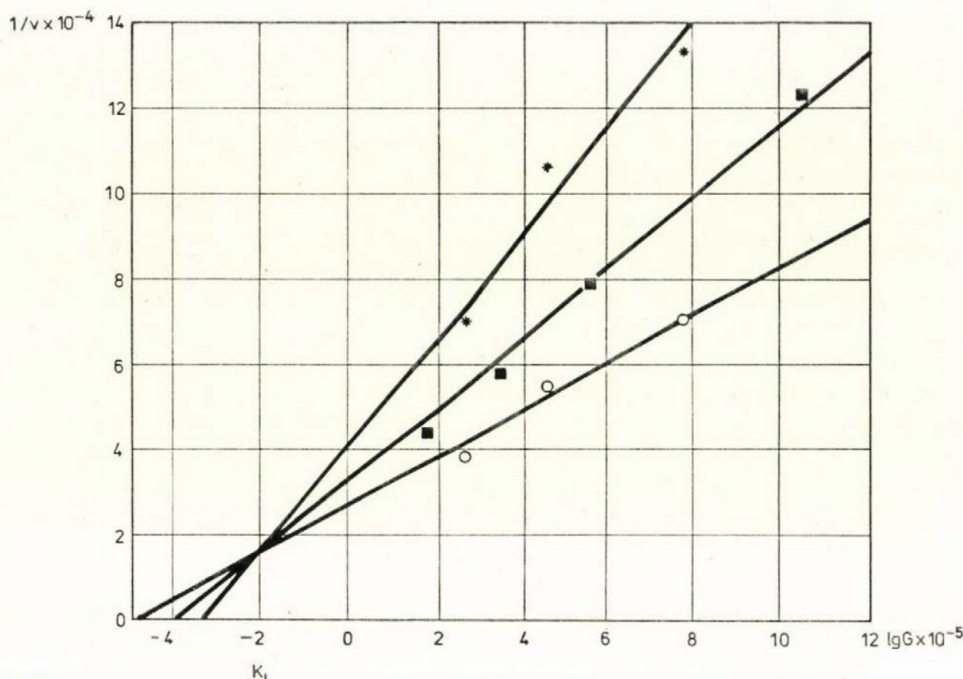


Fig. 2. Inhibition of porcine TPO activity by IgG isotype of anti-TPO antibodies according to Dixon. The inhibitor coefficient yields the values of 2.3×10^{-5} M by Dixon's diagram representing $1/v$ as a function of $[I]$ using different $[S]$. * 12 mM; ■ 20 mM; ○ 33 mM. Composition of the reaction mixture is given in the Methods; (v is calculated as $\text{nmol oxidized guaiacol} \times \text{min}^{-1} \times \text{mg protein}^{-1}$)

5. Comparing the inhibitory potency of IgG fragments on TPO activity the IgG Fc was ineffective but IgG F(ab')₂ inhibited the TPO activity, so the inhibition can be due to F(ab')₂ fragment.

6. In our experiment IgGs of 15 patients from 23 Hashimoto's patients inhibited the TPO activity using guaiacol method.

Discussion

The antigenic determinants on TPO molecule recognized by the autoantibodies are heterogeneous [19, 20] and are predominantly IgG1 and IgG4 subclasses [21, 22]. Furthermore, it has been reported that polyclonal anti-TPO antibodies bind to TPO, but are not able to modify the activity of enzyme [23]. Few workers reported that they not only bind TPO, but also inhibit the activity [13, 24–29]. Saller et al. [25] suggested the

use of a very rigorous approach of a possible inhibitory effect of anti-TPO antibodies. There are several misunderstandings in the literature which came from the selection of the reaction conditions (improper concentration range of the anti-TPO antibodies, the incorrect interpretation of degree of oxidation with iodide substrate).

Two authors described [24, 29] that serum or IgG partially ("dose-dependent") inhibited the enzymatic activity. In the present study we kinetically characterized the inhibition and found a competitive inhibition with guaiacol substrate, that has been completely cut out by anti-TPO antibodies from patients with hypothyroidism.

The opinion is not consistent regarding to the connection between the catalytic site (two second substrate binding sites) and the epitopes. Kohno et al. [13] recognized that in the hierarchy of epitopes on TPO molecule by autoantibodies may be hypothetical classified into four groups which might or might not be related to substrate combining sites (iodide- or tyrosyl residue-combining site). As also described by others the autoimmune response to TPO is known to be associated with a large number of autoantigenic epitop on TPO, including the enzymatic site [27, 28, 30]. They found that anti-TPO autoantibodies in AITD have been shown to be directed predominantly to the carboxyl terminal of the TPO molecule [31–33]. On the basis of our experiments we concluded that part of the autoantibodies may be attached to the binding site of the active centre, which binds aromatic donor molecules. The link between the epitopes and the active centre may derive from a spherical or purely competitive inhibition.

The anti-TPO antibodies which inhibited the TPO, measured from serum of AITD or GD [13, 25, 27], but others did not find any difference between serum from AITD and the normal control serum [26]. Our observation supports the idea concerning the inhibitory effect of anti-TPO on thyroid hormone production, since 15 IgGs from 23 patients with hypothyroidism inhibited the activity of TPO, and 19 IgGs inhibition determined chemiluminescence technique, $r=0.76$ (data not shown). Our prospective study using chemiluminescence method should provide further evidence for the possible pathogenic role of inhibitory type of anti-TPO antibodies in autoimmune thyroid disease.

Acknowledgements. This work has been supported by the Scientific Research Council, Hungarian Ministry of Welfare (023/94–96).

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SEROEPIDEMIOLOGIC STUDY OF ANTI-HAV IgG IN HEALTH-CARE WORKERS

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(Received December 13, 1994)

(Accepted December 19, 1994)

The seroprevalence of Hepatitis A virus IgG antibody (Anti-HAV IgG) was assessed in 410 health-care workers, included 97 physicians, 174 nurses, 57 auxiliary staff, 23 cleaning staff, and 59 from various professions. The overall seroprevalence rate was 50.2% with a mean age of 38.2 years, physicians 41.9%, nurses 48.6%, auxiliary staff 47.4% cleaning staff 87.0%, various professions 57.6%. A low seroprevalence was found in young employees, while a high seroprevalence increasing with age was demonstrated in older employees.

The low seroprevalence rate may be returned to improvements of the socioeconomic conditions in Hungary.

Hepatitis A is generally a mild and self-limited disease, resolving within two to four weeks. It passes asymptotically in 90% of infected individuals, and may take a severer course with increasing of the age [1, 5]. The seroprevalence of Hepatitis A IgG Antibodies (Anti-HAV IgG), usually is related to age and socioeconomic status [3, 6]. The seroprevalence may reach to higher than 90% of general population in areas of hyperendemicity of Hepatitis A Virus (HAV) infection [4, 12]. Occupational transmission of HAV infection has been documented in health-care workers [7, 8]. Recently, a new hepatitis A vaccine became commercially available in many countries [9]. In order to obtain a general view of the epidemiology of HAV infection in hospital employees, we carried out this study to determine the seroprevalence of Anti-HAV IgG.

Materials and methods

Study population. In the course of routine medical examination of László hospital employees during the period from 1986 till 1993, sera of some hospital workers were annually examined to presence of Anti-HAV IgG. Results of this test were collected only for staff still working in the hospital.

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The 410 persons examined represent 28.4% of the total number of hospital workers. A total of 61 men and 349 women, included 97 physicians, 174 nurses, 57 auxiliary staff, 23 cleaning staff and 59 persons of various professions. The subjects were stratified into four age groups, 18–30 years old, 31–40 years old, 41–50 years old, and over 51 years old.

Organon Teknika Microelisa, Roche Cobas-Core Automated ELISA assays were used to determine of Anti-HAV IgG. If we experienced seroconversion from Anti-HAV IgG negative to Anti-HAV IgG positive condition, we repeated the serological tests and carried out Anti-HAV IgM test, too. The tests were carried out as instructed by the manufacturer. The sera were stored at -20°C before investigation.

Statistical analysis of the data was performed by the chi-square test for assessment of P value.

Results

Out of the 410 sera tested, Anti-HAV IgG was detected in 206. Distribution of the seroprevalence according to sex of workers is illustrated in Table I. No statistical difference between men and women was observed. The overall seroprevalence was 50.2% to a mean age of 38.2 years.

Table I

Prevalence of anti-HAV IgG in hospital workers

	Examined No. (%)	Mean age (years)	Anti-HAV IgG positive No. (%)	P value
Men	61 (14.9)	44.0	32 (52.5)	NS*
Women	349 (85.1)	37.1	174 (49.9)	NS*

* Not significant

In Table II details on seroprevalence of Anti-HAV IgG of each professional group are shown. A significantly higher seroprevalence of Anti-HAV IgG was found in the cleaning staff group as compared with other professional groups. Also a slightly higher seroprevalence in the group of "others" than physicians, nurses, and auxiliary staff was observed. In nurses and auxiliary staff a non-significantly higher seroprevalence was shown than in physicians. A low seroprevalence of Anti-HAV IgG was noted in the 18–30 years group, while a higher frequency was shown in older groups increasing with age (Table III). Four cases a seroconversion were reported during the present study (2 physicians, 1 nurse, 1 auxiliary staff members). The annual incidence of seroconversion rate of Anti-HAV IgG was 121.9 ‰ on an average.

Table II*Prevalence of Anti-HAV IgG according to profession of hospital workers*

Profession group	Examined No.	Mean age (years)	Anti-HAV IgG positive No. (%)	P value
Physicians	97	41.9	40 (41.2)	NS*
Nurses	174	35.1	85 (48.6)	NS*
Auxiliary staff	57	36.9	27 (47.4)	NS*
Cleaning staff	23	45.0	20 (87.0)	<0.001
Others	59	39.6	34 (57.6)	<0.01

* Not significant

Table III*Prevalence of Anti-HAV (IgG) according to age of the hospital workers*

Age group (years)	Examined No.	Anti-HAV IgG positive No. (%)	P value
18-30	145	38 (26.2)	NS*
31-40	90	35 (38.9)	NS*
41-50	101	69 (68.3)	<0.001
>51	74	64 (86.5)	<0.01

* Not significant

Discussion and conclusions

In general, a distinct correlation was observed between prevalence and age in all groups studied. The high rate of seroprevalence in cleaning staff members may be associated with the fact that this group contains the oldest workers and, besides, these persons belong to low socioeconomic status. The reason for the slightly high seroprevalence in groups of "others" may be that most of these workers contact with infectious materials during in the work, in addition to their low socioeconomic status. Nurses and auxiliary staff members were more frequently Anti-HAV IgG positive than

physicians, probably attributable to the fact that they have a more occupational exposure to patients and infectious materials than the latter.

Results of the present study do not differ appreciably from data obtained in Western Europe [10, 11], but are low in comparison even with general seroprevalence in Eastern European countries [4, 12]. Our results may reflect improvements of socioeconomic conditions in Hungary in the last decades.

Acknowledgements. The authors kindly thank the staff in the Virological Laboratory for their help in performing the tests.

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COMPARISON OF SELECTED SPECIES OF *BIPOLARIS*, *DRECHSLERA* AND *EXSEROHILUM* BY RANDOM AMPLIFICATION OF POLYMORPHIC DNA

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(Received December 20, 1994)

(Accepted January 23, 1995)

Forty-six strains representing 15 species of *Drechslera*, five of *Bipolaris* and four of *Exserohilum*, as well as two formae of *Drechslera teres* were compared by RAPD analysis. *Drechslera* formed a large, heterologous group, while species of *Bipolaris* and *Exserohilum* were more closely related. Strong pair-wise affinities were observed between *D. graminea* and *D. teres*, *D. tritici-repentis* and *D. bromi*, *D. siccans* and *D. biseptata*, *D. fugax* and *D. poae*, *B. sorghicola* and *B. zeicola*, as well as between *E. rostratum* and *E. turcicum*.

The anamorphic hyphomycete group known as '*Helminthosporium*' sensu lato included over 100 species associated with various disease symptoms on wild and cultivated members of *Poaceae*. The morphological heterogeneity of these graminicolous fungi necessitated the revision of their classification [1–6]. It has been proposed by these and other workers that three genera, *Bipolaris*, *Drechslera*, and *Exserohilum* can be recognized within this diverse group. The distant teleomorphic connections (*Cochliobolus*, *Pyrenophora* and *Setosphaeria*) reflect the great phylogenetic differences among the three anamorphic genera, but the morphological, geographical and ecological variability among species of the same genus also calls for further studies on infrageneric relationships. Moreover, additional species and new combinations, worthy of comparative investigations have been described in the last few years [7–9].

Random amplification of polymorphic DNA (RAPD) has increasingly been used for the determination of phylogenetic relationships and for the estimation of genetic diversity within fungal populations [10–14]. In this study, we identified nearly 300

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RAPD markers and examined variations in the RAPD patterns of 46 different strains representing 24 species and two forms of the genera *Bipolaris*, *Drechslera*, and *Exserohilum*. The relationships of these fungi were summarized in phylogenetic trees.

Materials and methods

Strains. Forty-six strains representing 15 species of *Drechslera*, five of *Bipolaris* and four species of *Exserohilum*, as well as two formae of *Drechslera teres* were collected in Hungary by the authors or were kindly provided by Drs. J. L. Alcorn (Department of Primary Industries, Agricultural Research Laboratories, Indooroopilly, Queensland, Australia), A. Lichon (South Australian Department of Agriculture, Waite Agricultural Research Institute, Glen Osmond, Australia), and P. Wolf (Technische Universität München, Lehrstuhl für Phytopathologie, Freising-Weihenstephan, Germany). Sources and origin of these strains are given in Table I.

DNA isolation. Fungi were grown on a complete medium (CM, salts of Czapek solution, glucose, 20 g; yeast extract, peptone and casein hydrolysate, 3 g of each per litre) in 500 ml volume inoculated with 10^6 conidia and incubated on a rotary shaker for 45–48 h at 120 r.p.m. at 25 °C. In the case of non-sporulating strains the inoculum consisted of young, minced mycelium. The mycelium was harvested by filtration, washed several times with sterile cold water, blotted dry and freeze-dried. Mycelium (0.2 g) was powdered in liquid nitrogen, extracted in 2 ml lysing buffer (50 mM TrisHCl, 50 mM EDTA, 2% (w/v) N-laurylsarcosine (Sigma, St. Louis, USA) and 1% (v/v) 2-mercaptoethanol) for 2 h at 65 °C, centrifuged to remove cell debris; the supernatant was extracted sequentially with equal volumes of phenol, phenol - chloroform - isoamyl alcohol (25:24:1), chloroform - isoamyl alcohol (24: 1) and diethylether. The DNA was precipitated with 3 M sodium acetate and 2 vol ethanol, washed in 70% (v/v) ethanol, and dissolved in 200 µl distilled water. This material was further purified with RNase, 200 µg ml⁻¹ (Sigma) then with Proteinase K, 200 µg ml⁻¹ (Serva, Heidelberg, Germany). DNA was again precipitated and dissolved in 50 µl distilled water.

RAPD analysis. Six 10-mer oligonucleotides of a RAPD-primer kit obtained from Operon Technologies (Alameda, California, USA) were chosen. Nucleotide sequences of these primers were: OPE-01 (CCCAAGGTCC), OPE-03 (CCAGATGCAC), OPE-07 (AGATGCAGCC), OPE-15 (ACGCACAACC), OPE-19 (ACGGCGTATG), OPE-20 (AACGGTGACC). Amplification reactions were carried out in 50 µl volumes containing 12.5 ng fungal DNA, 10 pmole of primer, 50 µM each of dCTP, dGTP, dATP and dTTP (Boehringer, Mannheim, Germany), 1 unit of Taq DNA polymerase (Amersham International, Amersham, UK) and 5 µl Amersham-buffer under a drop of mineral oil. A Perkin-Elmer Cetus thermal cycler was used for amplification with the following cycling programme: initial denaturation at 92 °C for 5 min, 35 cycles of 1 min at 93 °C, 1 min at 36 °C, 2 min at 72 °C, and a final incubation at 72 °C for 7 min. After polymerase chain reactions (PCR) 12 µl aliquots of the products were electrophoresed in 1.5 % agarose gel. Gels were stained with 0.5 µg ml⁻¹ of ethidium bromide, visualized and photographed under UV illumination at 312 nm. *Pst*I-digested λ phage DNA was used as a size marker.

Data analysis. RAPD patterns were analysed by scoring 1 for the presence and 0 for the absence of the bands appearing at the same position in repeated runs. The data were grouped in a matrix and phylogenetic trees were constructed by the 'MIX' and the 'DRAWGRAM' programmes of PHYLIP 3.5c developed by J. Felsenstein. Within 'MIX' the Camin-Sokal parsimony method was chosen.

Results

The rarity of molecular taxonomical studies has prompted us to investigate DNA polymorphisms among different species and strains of *Bipolaris*, *Drechslera* and *Exserohilum* by RAPD analysis [15]. In this method DNA polymorphisms are generated by enzymatic amplification of genomic DNA fragments using the polymerase chain reaction (PCR) with synthetic 10-mer oligonucleotide primers. Five of the six primers, namely OPE-03, OPE-07, OPE-15, OPE-19 and OPE-20 proved to be suitable for differentiation. The number of the amplification products per primer ranged from 0 to 10 with sizes between 0.2 and 3.83 kb. Only those bands were scored as RAPD markers that were always present in the same position and showed similar staining intensity in repeated runs of DNA samples of the same strain. As an example, Fig. 1 shows the high level of polymorphisms obtained among various species of the three genera. Altogether 298 markers were scored to assess the genetic variability among these fungi.

Table I

Names, code numbers and origin of *Drechslera*, *Bipolaris* and *Exserohilum* strains

No./Species	Strain number	Other codes	Origin	Collector
1 <i>D. avenacea</i>	BRIP 13049	DAR 33699	oat, AUS	B. Semple
2 <i>D. biseptata</i>	PA-125	—	wheat, H	own isolate
3 <i>D. bromi</i>	PA-74	—	<i>Bromus erectus</i> , H	own isolate
4 <i>D. dictyoides</i>	BRIP 12833	LEV 11079b	wheat, NZ	J. G. Hampton
5 <i>D. erythrospila</i>	BRIP 12968	30	<i>Agrostis</i> sp., AUS	M. Mebalds
6 <i>D. fugax</i>	BRIP 12999	655	<i>Agrostis tenuis</i> , Canada	A. Hagan
7 <i>D. graminea</i>	BRIP 6515	DAOM 159452	barley, Canada	T. G. Atkinson
8 <i>Pyrenophora hordei</i> (<i>Drechslera anamorph</i>)	—	—	barley, AUS	H. Wallwork
9 <i>D. tuberosa</i>	BRIP 12914	WU 51/78	<i>Hordeum murinum</i> , NZ	J. E. Sheridan
10 <i>D. phlei</i>	BRIP 6506	DAOM 124884	<i>Phleum pratense</i> , Canada	W. Berkenkamp
11 <i>D. poae</i>	BRIP 12768	DAOM 169240	<i>Poa pratensis</i> , Canada	M. Barkworth
12 <i>D. siccans</i>	BRIP 12338	5066	<i>Lolium multiflorum</i> , NZ	E. H. C. McKenzie
13 <i>D. teres</i>	BRIP 15703	8712	barley, AUS	J. L. Alcorn
14 <i>D. teres</i> f. <i>maculata</i>	01/2T	—	barley, Germany (P. Wolf)	—
15 <i>D. teres</i> f. <i>maculata</i>	II/2	—	barley, Germany (P. Wolf)	—
16 <i>D. teres</i> f. <i>maculata</i>	PA-117.1/1	—	barley, H	own isolate
17 <i>D. teres</i> f. <i>teres</i>	04/6T	—	barley, Germany (P. Wolf)	—
18 <i>D. teres</i> f. <i>teres</i>	140.2	—	barley, Germany (P. Wolf)	—
19 <i>D. teres</i> f. <i>teres</i>	PA-103.5/11	—	barley, H	own isolate
20 <i>D. teres</i> f. <i>teres</i>	PA-115.1/1	—	barley, H	own isolate

Continued on p. 358

Table I continued

21 <i>D. teres f. teres</i>	PA-115.1/2	—	barley, H	own isolate
22 <i>D. teres f. teres</i>	PA-115.2/1	—	barley, H	own isolate
23 <i>D. teres f. teres</i>	PA-115.9/2	—	barley, H	own isolate
24 <i>D. teres f. teres</i>	PA-117.1/2	—	barley, H	own isolate
25 <i>D. teres f. teres</i>	PA-117.5	—	barley, H	own isolate
26 <i>D. teres f. teres</i>	PA-117.12	—	barley, H	own isolate
27 <i>D. teres f. teres</i>	PA-119.2	—	barley, H	own isolate
28 <i>D. tritici-repentis</i>	BRIP 12622	21615	wheat, AUS	R. G. Rees
29 <i>D. wirreganensis</i>	—	—	barley, AUS	H. Wallwork
30 <i>B. cynodontis</i>	BRIP 16821	—	<i>Digitaria smutsii</i> , AUS	Wal Scattini
31 <i>B. cynodontis</i>	PA-111	—	<i>Cynodon dactylon</i> , H	own isolate
32 <i>B. sorghicola</i>	BRIP 12154	7791	<i>Sorghum bicolor</i> , AUS	P. E. Mayers
33 <i>B. sorokiniana</i>	PA-0	—	barley, H	own isolate
34 <i>B. sorokiniana</i>	PA-13	—	<i>Bromus erectus</i> , H	own isolate
35 <i>B. sorokiniana</i>	PA-28	—	<i>Poa pratensis</i> , H	own isolate
36 <i>B. sorokiniana</i>	PA-33	—	<i>Hordeum murinum</i> , H	own isolate
37 <i>B. sorokiniana</i>	PA-110	—	barley, H	own isolate
38 <i>B. sorokiniana</i>	PA-113	—	rye, H	own isolate
39 <i>B. sorokiniana</i>	PA-122	—	<i>Agropyron repens</i> , H	own isolate
40 <i>B. sorokiniana</i>	PA-124.2	—	wheat, H	own isolate
41 <i>B. victoriae</i>	BRIP 16739	HV 408	oat, USA	C. F. Zimmerman
42 <i>B. zeicola</i>	BRIP 16102	8804-2	<i>Cymbopogon citratus</i> , AUS	D. E. Shaw
43 <i>E. monoceras</i>	RIP 12236	—	<i>Echinochloa colona</i> , AUS	M. Vincent
44 <i>E. pedicellatum</i>	PA-126	—	maize, H	own isolate
45 <i>E. rostratum</i>	BRIP 15876	8786b	<i>Eleusine indica</i> , AUS	J. L. Alcorn
46 <i>E. turcicum</i>	BRIP 13326	22233	<i>Sorghum sudanense</i> , AUS	R. Jones

Isolates Nos 1, 4, 5, 6, 8, 10, 11, 13, 14, 16, 31, 35, 37, 46, 47, 48, 50, 51 and 60 were sent by J. L. Alcorn, Nos 9 and 34 were provided by A. Lichon, Nos 17, 18, 20 and 21 were sent by P. Wolf.
AUS - Australia, H - Hungary, NZ - New Zealand

When the matrix containing all the RAPD markers obtained for the 46 strains was first viewed, remarkable differences were recognized especially between *Drechslera* and *Bipolaris* and several pairs of species were found without a single common marker. This made it impossible to arrange all species in a single phylogenetic tree, therefore a separate tree for *Drechslera* was first constructed, then another for *Bipolaris* and a third common one for *Bipolaris* and *Exserohilum*. Finally, an independent tree was drawn to assess the relationships among 14 isolates of *Drechslera teres f. maculata* and *Drechslera teres f. teres*, as well as single isolates of each of *Drechslera graminea* and *Drechslera teres*. Figure 2 shows the phylogenetic relationships of *Drechslera* species. As can be seen there are close pair-wise affinities between *D. graminea* and *D. teres*, *Drechslera tritici-repentis* and *Drechslera bromi*, *Drechslera siccans* and *Drechslera biseptata*, as well as between *Drechslera fugax* and *Drechslera poae*. These pairs of species attracted

further ones, such as *Pyrenophora hordei* (with *Drechslera* anamorph) and *Drechslera phlei*, *Drechslera wirreganensis*, *Drechslera avenacea* and *Drechslera dictyoides* and *Drechslera erythrospila*, respectively. Altogether four definite subgroups were identified within *Drechslera*, the only species that remained in this comparison without close relatives was *Drechslera tuberosa*.

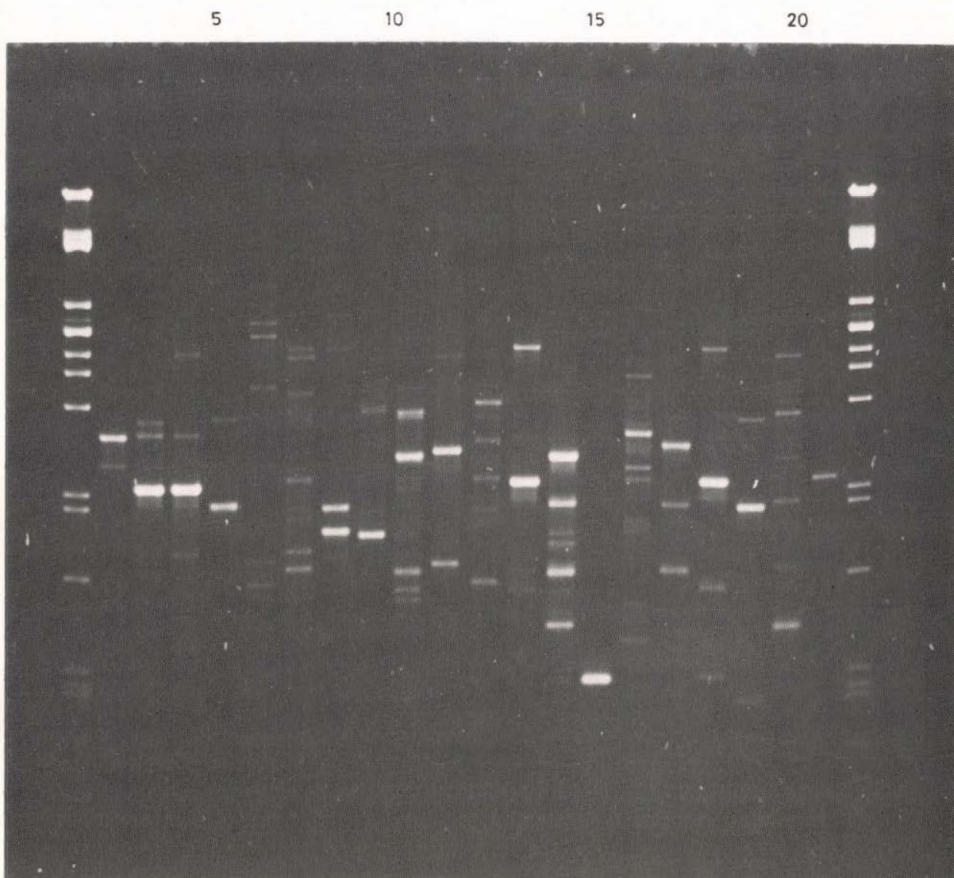


Fig. 1. Random amplification of polymorphic DNA from 20 isolates of *Bipolaris*, *Drechslera* and *Exserohilum* using the OPE-07 primer. Lanes are: 1 - λ ladder, 2 - *D. wirreganensis*, 3 - *B. zeicola*, 4 - *B. sorghicola*, 5 - *D. avenacea*, 6 - *D. erythrospila*, 7 - *D. bromi*, 8 - *E. pedicellatum*, 9 - *D. fugax*, 10 - nonsporulating *Drechslera* (excluded), 11 - *D. phlei*, 12 - *D. poae*, 13 - *B. sorokiniana* (PA-113), 14 - nonsporulating *Drechslera* (excluded), 15 - *D. siccans*, 16 - *E. rostratum*, 17 - nonsporulating *Drechslera* (excluded), 18 - *B. sorokiniana* (PA410), 19 - *D. tuberosa*, 20 - *E. monoceras*, 21 - *E. turcicum*, 22 - λ ladder

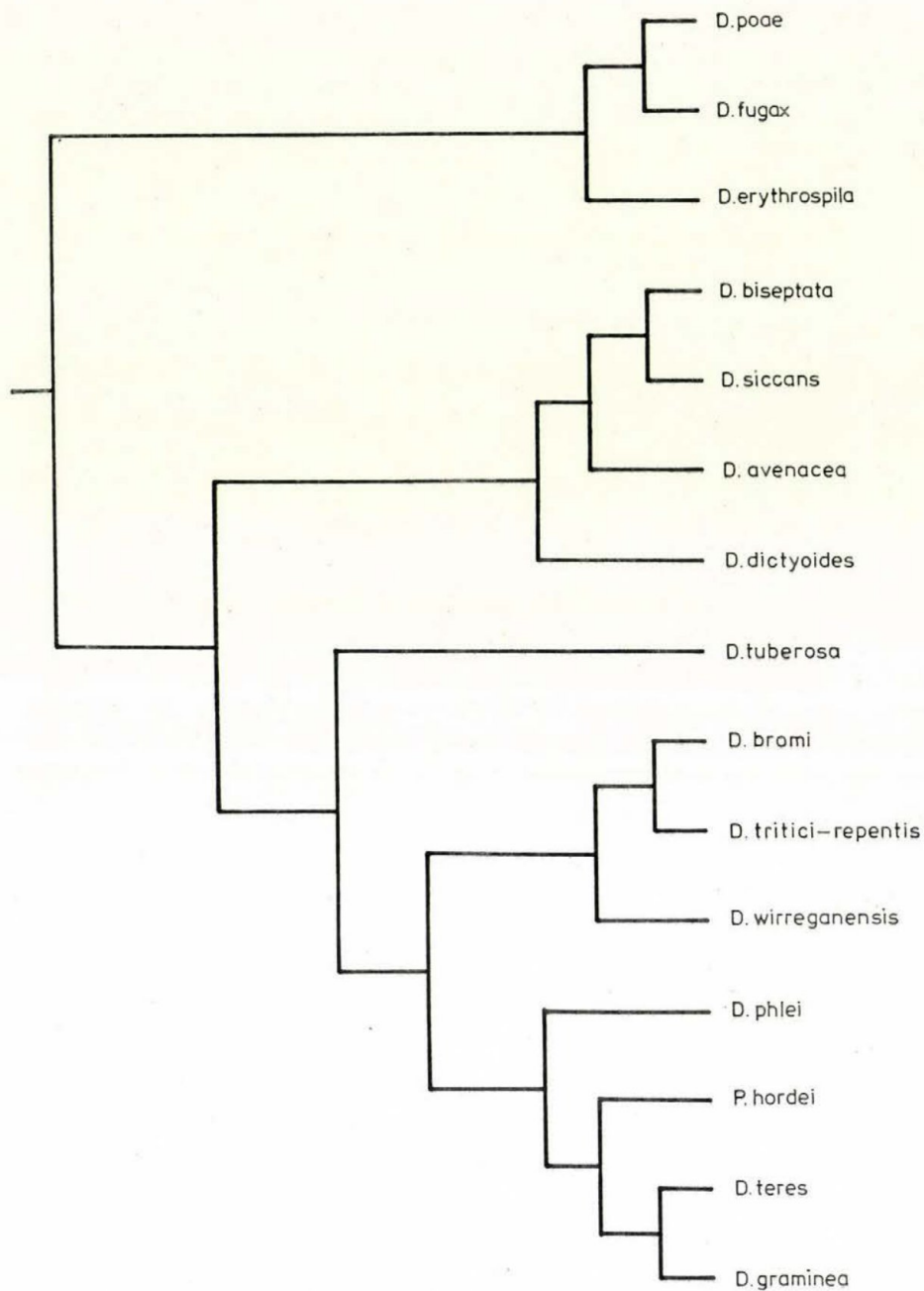


Fig. 2. Relationships among *Drechslera* species as estimated by random amplification of polymorphic DNA

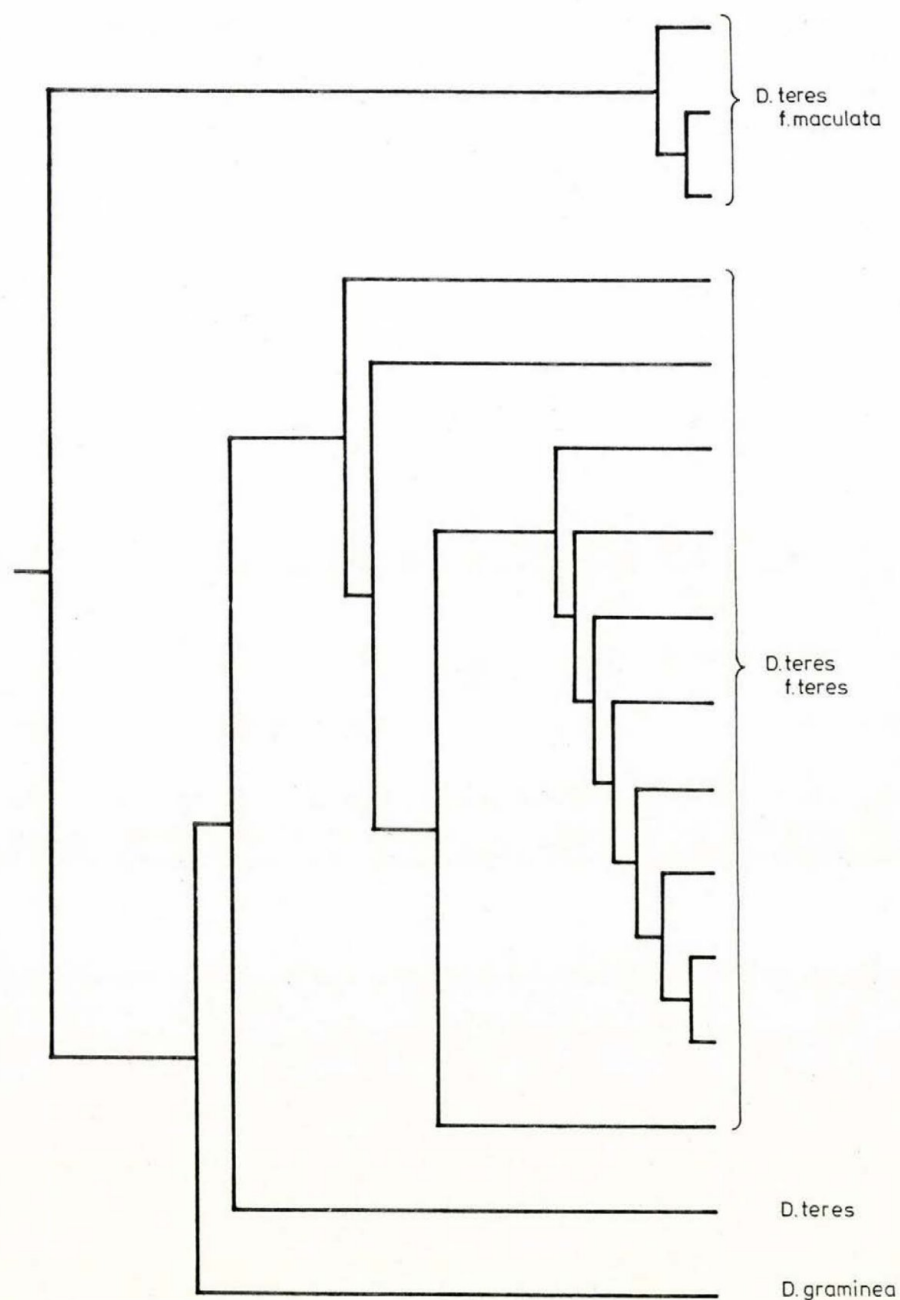


Fig. 3. Relationships among formae of *D. teres* and *D. graminea* as estimated by random amplification of polymorphic DNA

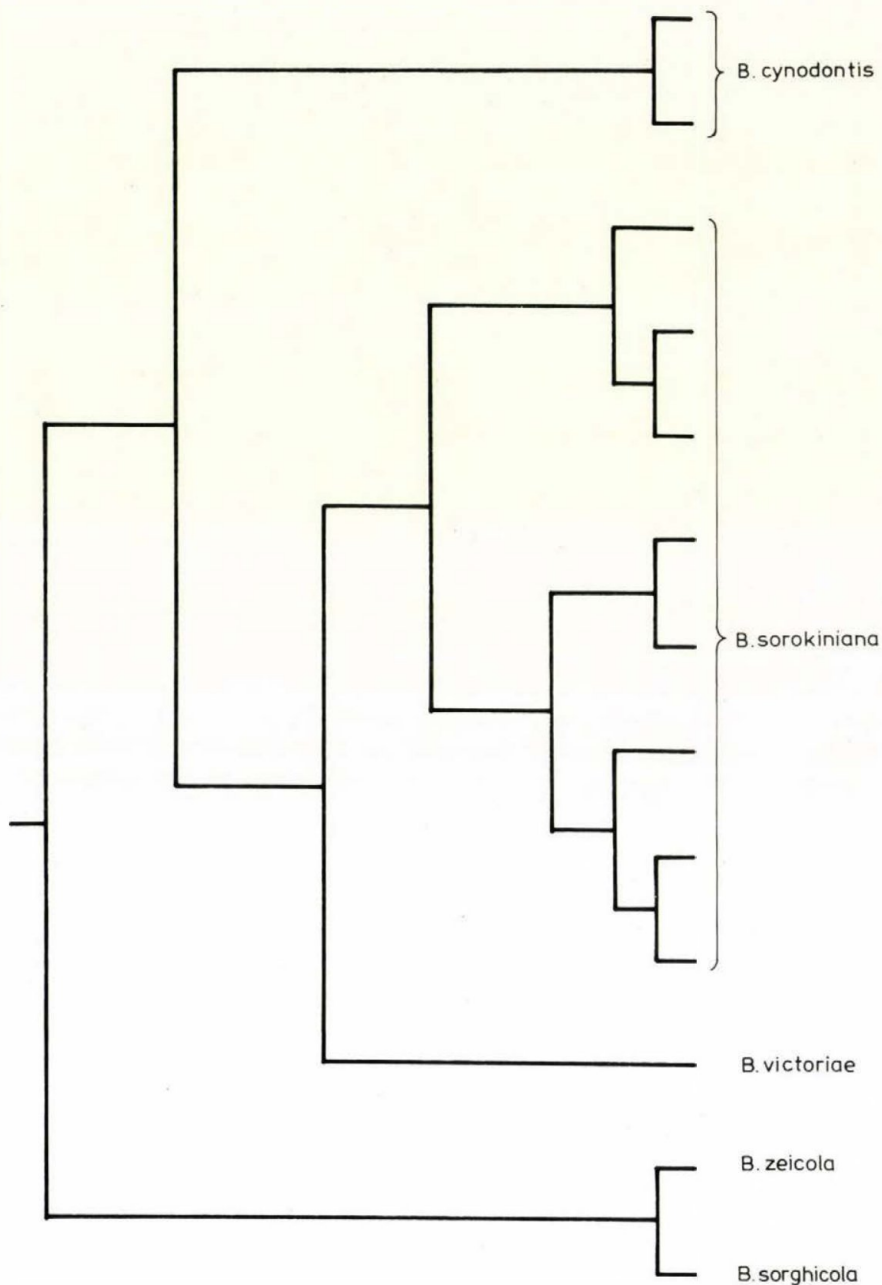


Fig. 4. Relationships among *Bipolaris* species as estimated by random amplification of polymorphic DNA

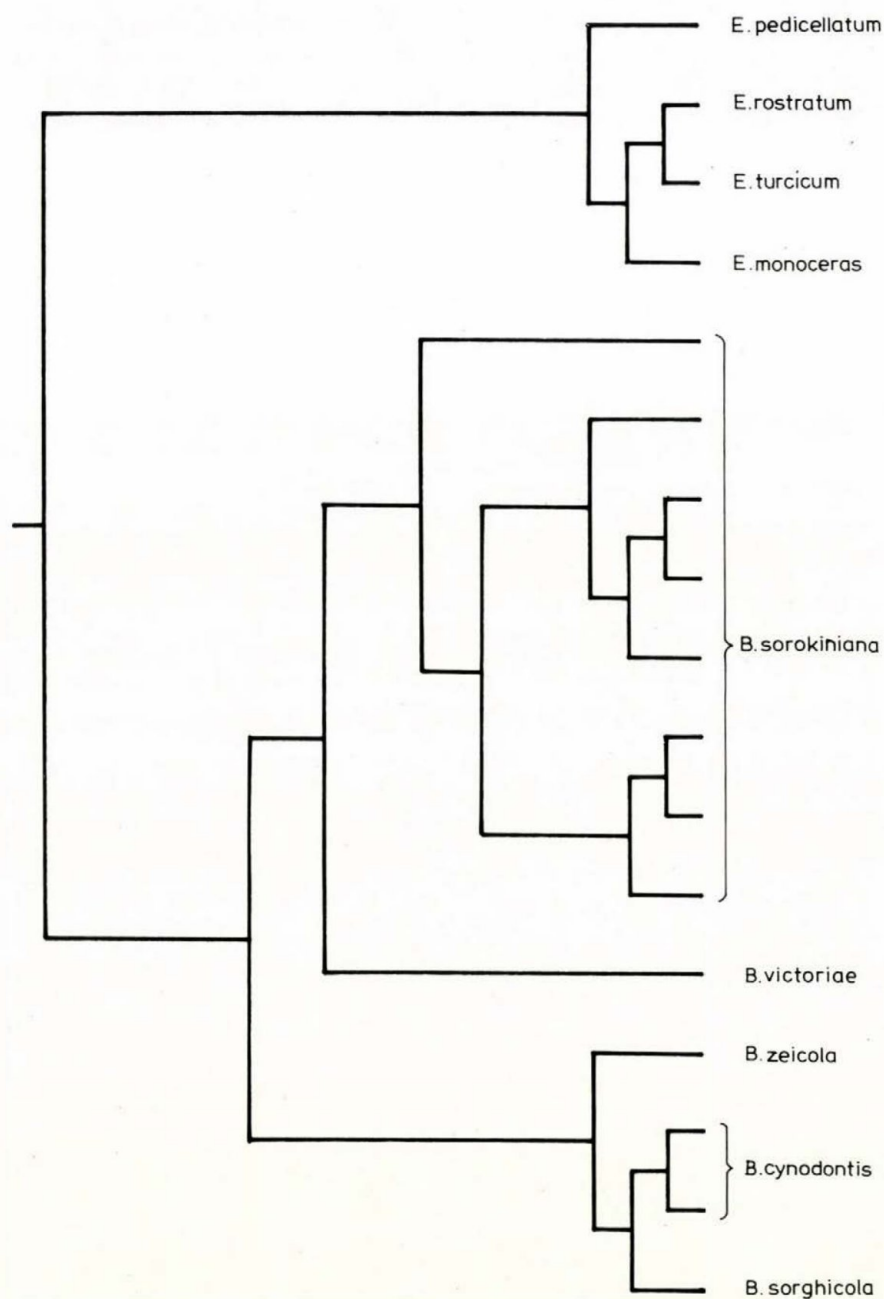


Fig. 5. A common phylogenetic tree for *Bipolaris* and *Exserohilum*

The *D. graminea*-*D. teres* complex was represented by 16 isolates collected from barley. Isolates that belonged to f. *teres* and f. *graminea*, respectively, were clustered into two distinct groups, while *D. teres* strain BRIP 15703, which was received without further specification maintained its distinctness and the same position was true for *D. graminea* (Fig. 3).

Grouping of *Bipolaris* species resulted in a clearly arranged tree (Fig. 4). A large central cluster was formed by *Bipolaris sorokiniana* isolates collected from various hosts. According to the expectations, a close relationship was found between the two strains of *Bipolaris cynodontis* (Marignoni) Shoem., that were isolated from *Digitaria smutsii* and *Cynodon dactylon* and an unambiguous affinity was also observed between *Bipolaris sorghicola* and *Bipolaris zeicola*.

Because of the small number of individuals in *Exserohilum*, the construction of a separate tree for these fungi was unreasonable. Moreover, species of this genus shared many common RAPD markers with *Bipolaris*, therefore a common tree was drawn to present the proper relationships. As it appears in Fig. 5 the four species of *Exserohilum* formed a distinct cluster, supporting the separation of the two genera. However, worthy to mention that on the basis of RAPD analysis stronger affinities were found between certain members of *Exserohilum* and *Bipolaris*, than among distant species within *Drechslera*. At the same time, when *Exserohilum* species were grouped together with *Bipolaris*, this caused some rearrangements within the latter, that was manifested in the appearance of a new cluster formed by *B. sorghicola*, *B. cynodontis* and *B. zeicola*.

Discussion

Both numerical and molecular approaches have increasingly been used to provide additional information on the taxonomy of selected species in *Bipolaris*, *Drechslera* and *Exserohilum*. In an early experiment ribosomal proteins from *Bipolaris maydis*, *Exserohilum turcicum*, *B. sorghicola* and *B. sorokiniana* were compared [16]; great differences were found among genera and much lesser ones among species of the same genus. Isozyme polymorphisms were evaluated among races of *B. zeicola* (teleomorph: *Cochliobolus carbonum*) and although considerable similarities were established between race 1 and race 2, race 3 isolates of the fungus could easily be distinguished by the presence of specific esterase and leucine aminopeptidase bands [17].

PCR based nucleic acid techniques have also been applied to assess similarities or differences among certain members of this group of fungi. By using 15–21-mer oligonucleotide primers to generate DNA polymorphisms among five species of *Drechslera*, Bulat and Mironenko [18] demonstrated significant differences among *D. tritici-repentis*, *D. bromi* and *D. dictyoides* and complete identity among 12 isolates of *D. teres* and *D. graminea*. Jones and Dunkle [19] differentiated races of *B. zeicola* from one another and from certain other *Bipolaris* species (*B. maydis*, *Bipolaris victoriae*, *B. sorokiniana* and *Bipolaris oryzae*), as well as from *E. turcicum*. The arbitrary primers easily distinguished the species, but the four pathogenic races of *C. carbonum* proved to be very similar to one another in this test. However, specific primers, homologous to

short sequences of the *Tox2*, a gene coding for HC-toxin production was used, race 1 isolates were easily differentiated from the other races.

Among the numerical studies an attempt of Lam and Chapman [20] gave the most important contribution to the taxonomy of these fungi. When 48 isolates of four *Drechslera* species were compared on the basis of symptoms, sporulation, as well as conidium and colony morphology a new species, *Drechslera andersenii* Lam was separated and this taxon is still accepted as a valid species. A pioneer work of Ibrahim and Threlfall [21] is worth mentioning. In their study morphological properties, pathological features and neomycin-sensitivity of 23 '*Helminthosporium*' species were subjected to numerical analysis. The major conclusions of this work were, that *Drechslera*, *Bipolaris* and *Stemphylium* should be treated as separate genera, and "*Helminthosporium siccans*" (*D. siccans*) and "*Helminthosporium avenaceum*" (*D. avenacea*) were suggested to be conspecific taxa.

As far as our results are concerned, the strong affinity of *D. graminea* and *D. teres* found by us is supported by earlier genetical studies [22] and recent molecular analyses [23]. The similarities of the RAPD patterns found between *D. tritici-repentis* and *D. bromi*, *D. avenacea* and *D. siccans*, as well as *D. fugax* and *D. poae* are in agreement with the morphological similarities of these pairs of fungi. On the other hand, a more pronounced distinction of *D. biseptata* was originally expected, as this species produces unusually small conidia with a few septa and its morphology greatly differs from that of the other *Drechslera* species. The infraspecific variability found within *B. sorokiniana* is quite reasonable, if the various host plant origins of the isolates are considered. Based on similarities in conidium morphology the close relationship found in the RAPD patterns of *B. victoriae* and *B. sorghicola* is not surprising and the affinity between *B. sorghicola* and *B. zeicola* is also reasonable in the light of reports on successful hybridization and ascospore production between the latter two species [22].

Acknowledgements. This research was supported from a Hungarian Scientific Research Fund (OTKA 1913/90). We thank J. L. Alcorn, A. Lichon and P. Wolf for providing laboratory strains. J. B. expresses his thanks to the foundation "Pro Agricultura Pannoniae" for travel grant. We also thank Dr. E. Barta for introducing us to the PHYLIP 3.5c programme and Kim Weldon for linguistic checking of the manuscript. The skillful technical assistance of T. Bagdány and G. Takács are appreciated.

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OXYGEN AND CARBON DIOXIDE REQUIREMENTS OF *HELICOBACTER PYLORI*

(A NOTE)

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(Received December 29, 1994)

(Accepted February 3, 1995)

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The oxygen and carbon dioxide requirements of 25 clinical isolates of *Helicobacter pylori* were studied in relation to pyruvate metabolism. A maximal number of 16 strains grew without added carbon dioxide under an atmosphere containing 6% oxygen and 0.04% ambient atmospheric carbon dioxide contrary to a generally accepted idea. Enzymes acting on pyruvate as a parameter of the ATP regeneration system were only pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) and pyruvate formate-lyase (EC 2.3.1.54). The microaerophilism may be partly due to the conversion of pyruvate to acetyl-CoA by the two anaerobic enzymes, although the organism requires oxygen.

Microaerophiles are organisms for which oxygen (O₂) is both beneficial and deleterious [1]. They require O₂ for the ATP-regenerating respiration, although they are inhibited by excess O₂ or its derivatives. Microaerophiles include certain pathogenic bacteria, such as *Treponema pallidum* [2], *Borrelia hermsii* [3], *Campylobacter jejuni* [1], and *Helicobacter pylori* currently suggested as a cause of non-specific chronic gastritis [4].

Although the isolation of *H. pylori* has been achieved using the same microaerobic technique as that for campylobacters [5], few studies have been made on the growing range of O₂ and carbon dioxide (CO₂) concentrations [6]. We studied the gaseous requirements by assessing total growth on plate cultures [7] and the microaerophilic nature by assaying enzymes acting on pyruvate as a parameter of the ATP regeneration system [8, 9].

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Materials and methods

Bacterial strains. *H. pylori* strains SM 1 to SM 25 were obtained from the antral biopsy specimens of patients in this hospital. They were isolated on Skirrow's medium (Eiken, Japan) using microaerobic techniques with CampyPak (BBL, USA), and were identified using the method of Itoh et al. [10].

Inocula and general culture conditions. Each strain was cultured on Brain-Heart Infusion Agar (BHIA; Difco, USA) plates with 7% horse blood and 0.25% yeast extract (Difco, USA) [2] at 37 °C for 3 days in an anaerobic jar containing 6% O₂, 10% CO₂ and residual nitrogen (N₂) provided by means of the method described below. The bacteria were suspended in a Brain-Heart Infusion Broth (Difco, USA) in accordance with the McFarland No. 1 turbidity standard (2×10^7 colony-forming units/ml as determined by the usual spread-plate technique) and 5 µl of the suspension was spot-inoculated onto the supplemented BHIA plates with a multi-inoculator (Sakuma, Japan). The inoculated plates were set in a humidifier or jar including a square of blotting paper containing water in order to maintain sufficient moisture [5], and then incubated at 37 °C for 3 days in different atmospheres as shown in Table I.

Gas atmospheres. Anaerobic incubation was performed in an anaerobic chamber, which had an atmosphere of 85% N₂, 10% CO₂ and 5% hydrogen [11]. Microaerobic incubation was performed using various O₂ concentrations with 0.04% ambient atmospheric CO₂ in jars, evacuated to 53, 93, 187, 293, 387, 480, 773, 867 hPa (40, 70, 140, 220, 290, 360, 580 and 650 mm of mercury, respectively) and then refilled with a gas mixture containing 99.96% N₂ and 0.04% CO₂ to give the O₂ levels of cca. 1%, 2%, 4%, 6%, 8%, 10%, 16% and 18%, respectively [7]. A candle producing cca. 17% O₂ and 3% CO₂ in the atmosphere [12] and a carbon dioxide (10%) incubator were used to estimate the CO₂ requirement. The bacterial growth was visually scored as good, moderate, poor or no growth [13]. All strains were tested at least three times.

Enzyme assays. Representative strains SM 6 (a strain requiring CO₂ as shown in Table I), SM 9, SM 17 and SM 24 (strains requiring no exogenous CO₂) were used for the enzyme assays. A three-day plate culture grown in a 6% O₂, 10% CO₂ and 84% N₂ atmosphere was suspended in a buffer suitable for each enzyme assay and harvested through centrifugation (8 000 g). The pellet was disrupted using a sonic oscillator (W-200P, Branson, USA) at 20 KHz for 10 min in a N₂ atmosphere. This extract was assayed for enzymes acting on pyruvate (Table II) using the methods of Lindmark and Müller [14], Abbe et al. (two enzymes) [15], Gorrell et al. [16], Cerkasovová et al. [17], Holtzclaw et al. [18] and Chang and Cronan, Jr. [19].

Results and discussion

Table I shows the O₂ and CO₂ requirements of the 25 isolates. Nine strains (36%) failed to grow in any O₂ concentrations with 0.04% CO₂ in the ambient atmosphere. The other 16 strains (64%) failed to grow under anaerobic conditions, the growth being accelerated with an increase in the O₂ concentration at a peak of 6% O₂. When the O₂ concentration surpassed 8%, the growth was reduced, being poor or absent at 16%, 18% O₂, and totally absent under aerobic conditions. All test strains grew well or moderately well in 10% CO₂ plus air, showing the best growth in 10% CO₂, 6% O₂ and 84% N₂. The candle jar was unable to support the growth of all the strains.

As for the enzyme activities, only pyruvate:ferredoxin oxidoreductase and pyruvate formate-lyase were detected in all the strains tested (Table II).

Table I

Oxygen and carbon dioxide requirements of 25 isolates of H. pylori

Degree of growth ^a	No. of strains grown under the indicated conditions												
	Anaerobic	O ₂ (%) ^b								Candle jar	10% CO ₂ plus air	10% CO ₂ plus 6% O ₂ , 84% N ₂	Aerobic
		1	2	4	6	8	10	16	18				
+++	0	0	0	9	11	2	1	0	0	8 (2)	14 (7)	25	0
++	0	0	8	3	3	10	5	0	0	10 (5)	11 (2)	0	0
+	0	9	3	3	2	2	8	6	1	2 (1)	0	0	0
-	25	16	14	10	9 ^c	11	11	19	24	5 (1)	0	0	25

^a +++, Good growth; ++, moderate growth; +, poor growth; -, no growth^b With 0.04% CO₂ the concentration of ambient atmospheric CO₂^c Presumed to be CO₂-requiring strains. Numbers of such strains are designated in parentheses

Table II

Activity of enzymes acting on pyruvate in H. pylori

Strain	Enzyme activity* (units)		
	Pyruvate:ferredoxin oxidoreductase (OD units/g protein/min)	Pyruvate formate-lyase (μmol of NADH/g protein/min)	Other enzymes acting on pyruvate
SM 6	+	(698)	(12)
SM 9	+	(593)	(4)
SM 17	+	(820)	(6)
SM 24	+	(445)	(2)

* Comprises pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), pyruvate formate-lyase (EC 2.3.1.54), pyruvate:lipate oxidoreductase (EC 1.2.4.1), pyruvate decarboxylase (EC 4.1.1.1), acetolactate synthase (EC 4.1.3.18), lactate dehydrogenase (EC 1.1.1.27), and pyruvate:ferricytochrome b₁ oxidoreductase (EC 1.2.2.2)

The principal gases affecting bacterial growth are O₂ and CO₂. We tested 25 isolates of *H. pylori* for their gaseous requirements, verifying that the organism was microaerophilic as reported by Marshall et al. [20]. The best growth occurred in the currently used atmosphere containing 6% O₂, where 16 isolates grew without the

addition of exogenous CO₂. Thus, not all *H. pylori* require CO₂ for growth in a low O₂ tension. However, an addition of 10% CO₂ could increase the maximum O₂ concentration permitting growth.

The bacterial responses to O₂ are related to the energy-producing mechanisms [9]. As the pyruvate metabolism usually represents the ATP-producing mechanisms, we measured the activity of seven enzymes acting upon pyruvate [1, 8, 9]: pyruvate:ferredoxin oxidoreductase, the O₂-sensitive pyruvate dehydrogenase of obligate anaerobes in the ferredoxin-dependent phosphoroclastic reaction [11]; pyruvate formate-lyase found in anaerobically-cultured facultative anaerobes such as *Enterobacteriaceae* [9]; pyruvate:lipoate oxidoreductase, a pyruvate dehydrogenase detected in aerobically-cultured facultative anaerobes and aerobes [16]; pyruvate decarboxylase, which is involved in alcoholic fermentation [17]; acetolactate synthase, which catalyses a reaction giving 2-acetyl-lactate from pyruvate by acetoin formation [18]; lactate dehydrogenase which catalyses a reaction in lactic acid fermentation giving lactate from pyruvate [15]; and pyruvate:ferricytochrome b₁ oxidoreductase, which catalyses the oxidative decarboxylation of pyruvate to acetic acid and CO₂ [19]. The two enzymes found in the *H. pylori* strains were pyruvate:ferredoxin oxidoreductase and pyruvate formate-lyase. Thus, *H. pylori* may be anaerobic in the pyruvate metabolism, although the organism did not actually grow in the absence of O₂. The reason may be that *H. pylori* has an incomplete phosphoroclastic reaction, not producing energy with the anaerobic ATP-producing metabolism alone. Being positive for cytochrome oxidase (ferrocyclochrome c: oxygen oxidoreductase, EC 1.9.3.1) [10], the organism utilizes oxygen as a terminal electron acceptor. It is, however, unable to grow under aerobic conditions, thereby suggesting the incompleteness of the respiratory chain, and is certainly lacking pyruvate:lipoate oxidoreductase which serves predominantly in the formation of acetyl-CoA to enter the tricarboxylic acid cycle present in all aerobic organism [9]. The microaerophilism of *H. pylori* may be caused by converting pyruvate to acetyl-CoA with the two anaerobic enzymes, although this organism does require O₂.

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MICROBIAL COUNTS AND CHARACTERISTICS OF *STREPTOVERTICILLIUM* STRAINS ISOLATED FROM SOILS IN THE JORDAN VALLEY

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(Received January 4, 1995)

(Accepted February 10, 1995)

A microbial survey of total bacterial count, agrobacteria, streptomycetes, and fungi was carried out in six locations in the Jordan Valley. The highest microbial counts for the four populations were in spring, the lowest mostly in autumn. No significant differences at $p < 0.05$ were observed either in the counts between the six locations, or in the seasonal counts of agrobacteria and streptomycetes between the six locations as well as within each location. The total bacterial counts (at $p < 0.01$) and fungi (at $p < 0.05$) differed significantly in the six locations. Microbial counts and the studied environmental conditions showed no significant regression. Of 552 streptomycetes strains isolated from soils collected from these locations, 58 belonged to the genus *Streptoverticillium*. They were distributed in five colour series grey (41%), red (31%), green (17%), blue (7%) and white (3%). Forty-eight strains had biverticillium spore bearing hyphae while only 10 had monoverticillium. While 28 strains produced distinctive reverse side pigment, only 8 and 6 strains produced soluble pigment and melanin pigment, respectively. Most strains utilized arabinose followed by fructose, mannitol, rhamnose, xylose, sucrose, inositol, and raffinose. Sixty-nine per cent of the strains exhibited activity in vitro test against *Pseudomonas aeruginosa*, and 45, 41, 28, and 7% against *Staphylococcus aureus*, *Bacillus cereus*, *Agrobacterium tumefaciens* and *Escherichia coli*, respectively.

Streptomycetes are known to be major antibiotic producing group among other microorganisms [1]. Therefore, various attempts were and are still being made to isolate them from various localities searching for novel antibiotics [2].

Many antagonistic microorganisms have also been studied as useful agents for the control of plant diseases [3]. Plant diseases caused by soil-borne pathogens are gaining more and more importance. Chemicals which are used to treat plant diseases have lost their attractiveness due to difficulties in their application, narrow and short-period effects, the appearance of resistant strains of pathogens, and economical considerations. Therefore, extensive studies on the biological control of plant pathogens in soil have been carried in recent years [4], particularly on *Streptomyces* [5].

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Soil remains the most important and fruitful source for the isolation of streptomycetes. The numbers and types of actinomycetes present in a particular soil will be greatly influenced by geographical location, soil temperature, soil type, soil pH, organic matter content, cultivation and moisture content.

It is widely recognized that success in isolating significant numbers of a particular actinomycete genus from soils can be highly dependent upon the choice of soils, but there are few quantitative data on the geographical and ecological distribution of actinomycetes [6]. Some actinomycete genera, such as *Streptomyces*, are widely distributed in soils throughout the world [2]. Other genera appear to have a more narrow distribution in nature [7]. Strains of *Streptovercillium* are rarely isolated from soils collected in the United States or England [8], but they have been frequently isolated from Indian soils.

Streptomycetes flora, in particular *Streptovercillium*, of Jordanian soils has not yet been studied. Therefore, a survey was started to study soil streptomycetes in Jordan in order to look for the possibility of finding some strains that can be studied as a mean of biological control of some plant diseases, e.g. crown gall disease or that can be used to produce novel antibiotics [9]. The present paper deals with the microbial count of viable total bacteria, agrobacteria, streptomycetes, and fungi, and the characteristics and antimicrobial activity of *Streptovercillium* strains isolated from fertile soils in the Jordan Valley.

Materials and methods

Area of study. Jordan Valley is the most important agricultural part in Jordan. Therefore, six regions along the Jordan River between Al Baqureh and Deir Alla have been chosen to collect soils from them.

Collection of soil samples. After removing surface litter the exposed soil was collected to a depth of 15 cm in sterile clean polyethylene bags. Equal aliquots of soil samples were collected from six fixed sites within each region in April, July, September and January (representing spring, summer, autumn, and winter, respectively), mixed thoroughly and sieved in a sieve (2 mm mesh). The sieved soil samples were placed in polyethylene bags, closed tightly and stored at reduced temperature. The polyethylene bags were opened from time to time to allow air exchange.

Microbial counts. Soil dilutions were made in sterile distilled water, then 0.1 ml of the appropriate dilutions was pipetted and spread evenly over the surface of agar plates with a sterile L-shaped glass rod. Triplicate plates of standard plate count agar (Merck), glycerol nitrate casein agar [10], malt extract agar (Merck) and D1 medium [11] were used for total viable bacterial count, streptomycetes count, fungal count and agrobacterial count, respectively. Plates were incubated at 27 °C for streptomycetes, fungi and agrobacteria; and at 37 °C for total viable bacteria and observed daily. The number of colonies was counted after 2–4 days for total bacteria, agrobacteria and fungi; and after 10 days for streptomycetes.

Isolation, purification and characterization of Streptomyces. Colonies which seemed to represent different types were transferred to oatmeal agar plates for further purification and characterization as described by Shirling and Gottlieb [12].

Soil analysis. Ten grams of soil was dried at 105 °C for 24 h in dry oven. Then the crucibles were cooled at room temperature and weighed to calculate the percentage of soil moisture. To calculate the percentage of organic matter the oven-dried soils were burned at 500 °C for 6 h. Then

the crucibles were cooled at room temperature and weighed again. To measure pH, soil solutions were prepared in water (1:2.5 w/v) [9].

Production of antibacterial agents. The *Streptoverticillium* isolates were grown on oatmeal agar at 27 °C for 14 days. Then agar discs of 9 mm diameter were placed on nutrient agar plates inoculated with *Staphylococcus aureus*, *Escherichia coli*, *Bacillus cereus*, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*. Inhibition zones were checked after 24 and 48 hours of incubation at 28 °C for *A. tumefaciens* and 37 °C for the others [9].

Results and discussion

Soil microflora, especially bacteria, fungi and streptomycetes are affected by many factors [13].

During the period of this study the highest microbial counts in all studied fields occurred as during spring while the lowest counts varied from field to field and depended on microorganism (Table I). Estimates of microbial counts vary according to the means of determination.

Plate counts usually give values ranging from few hundred thousands up to 200 million bacteria per gram of oven-dried soil, the abundance being a reflection of many environmental factors affecting these inhabitants. Bacterial and other microorganisms may exhibit associations with particular soil types. The environmental factors that characterize different soil types favour the development of particular microbial populations. In this study the average microbial count ranged between 10^6 and 10^9 per one gram dry weight of soils for bacteria, from 10^5 – 10^7 for streptomycetes, and from 10^3 – 10^7 for fungi and agrobacteria. This is located within the range of counts reported by many investigators [14–16]. However, differences in microbial counts in soils might be due to many factors either working alone or in combination such as climatic region, soil treatment, plant type and age, organic matter, soil moisture and methods of counting microorganisms as well as rotation of crops in the same soil. The low level of *Streptomyces* in the studied soils in Jordan might be due to the low content of organic matter in these soils (around 1.5%), since these organisms colonize soil rich in organic matter more easier than other soils with low content of organic matter due to the nature of their hyphal growth. Jakubczyk [17] studied the effect of dead plant material decomposition on the number of bacteria, fungi and actinomycetes and found a rather strong variation in their numbers.

Soil analysis showed similarity in organic matter content (around 1.5%), pH (around 7), and soil moisture (5%) in all studied fields. The highest mean seasonal temperature was in summer ranging between 29 and 31 °C, and the lowest was in winter ranging from 15 to 17. Multiple regression analysis of the populations showed no significant regression between their counts and these edaphic factors. Also no significant differences at $p < 0.05$ were observed in the counts of these microbial populations between the studied fields.

A total of 552 *Streptomyces* isolates were collected from all samples during the four seasons. In general, *Streptomyces* isolates were small, with discrete and leathery smooth surface but later developed a web of aerial mycelium that appeared granular, powdery and velvety. The isolates were classified into seven series according to the

colour of their mature sporulated aerial mycelia. The distribution of these isolates in the different colour series is shown in Table II. Based on the morphology of spore bearing hyphae, 58 of these isolates belonged to the genus *Streptoverticillium*.

Table I

Viable count fluctuation of total bacteria, streptomycetes, agrobacteria and fungi

	Sites					
	1	2	3	4	5	6
Total count ($\times 10^7$)						
spring	189	4	1451	7	702	1097
summer	1	3	1	5	30	9
autumn	0.4	0.5	1	0.4	3	1
winter	4	1	3	3	3	3
Streptomyces count ($\times 10^4$)						
spring	248	112	64	85	47	490
summer	75	114	59	26	42	16
autumn	31	81	38	13	46	17
winter	61	55	64	43	43	203
Agrobacterial count ($\times 10^4$)						
spring	95	17	169	75	400	139
summer	65	38	44	9	195	53
autumn	52	51	46	26	43	103
winter	148	49	25	78	52	68
Fungal count ($\times 10^4$)						
spring	205	62	48	87	1067	1542
summer	17	4	25	52	130	35
autumn	31	14	20	0.4	60	16
winter	10	9	20	25	18	17

Some actinomycete genera, such as *Streptomyces*, are widely distributed in soils throughout most of the world [2]. Strains of *Streptoverticillium* are rarely isolated [8]. Our *Streptoverticillium* isolates were further characterized. The distribution and characteristics of these isolates are shown in Table II. *Streptomycetes* are known to produce antibiotics in synthetic media; it has been possible to demonstrate its production in sterilized and amended soils [18]. Soulides [19] suggested that the frequency of streptomycin resistant strains in some soils indicated that they are exposed to this antibiotic. Al-Rashidi [20] attributed the failure of *Rhizobium japonicum* serotype 135 to colonize in soils of Abou-Ghraib to the activity of two isolates of *Streptomyces* common in these soils. As shown in Table II, part of the *Streptoverticillium* strains

isolated in this study exerted activity against *P. aeruginosa*, *S. aureus*, *B. cereus*, *A. tumefaciens* and *E. coli*. Nothing is known about the ecology of *Streptomyces* and *Agrobacterium* in Jordanian soil. The susceptibility of *A. tumefaciens* to antibiotics as indicated in this report must be taken into account when attempts are made to protect plants against the crown gall disease. Also, the occurrence of active isolates in these soils presents a potential for industrial exploitation of these isolates for antibiotic production. However, these results encourage further investigation to determine factors that favour the growth of strains antagonistic to the crown gall agent.

Table II

Distribution of Streptomyces and Streptovercicillium isolates in 7 colour series, and other characteristics of Streptovercicillium isolates

	Colour series							Total
	White	Gray	Yellow	Red	Blue	Green	Violet	
Streptomyces	32	256	34	80	54	64	32	552
%	5.8	46	6	14.5	9.8	11.6	5.8	100
Streptovercicillium	2	24	0	18	4	10	0	58
	3	41	0	31	7	17	0	100
Characteristics of <i>Streptovercicillium</i> isolates according to colour series								
Production of pigments								
Melanin	0	0	0	2	4	0	0	6
%	0	0	0	11	100	0	0	10
Soluble pigment	2	4	0	2	0	0	0	8
%	100	17	0	11	0	0	0	14
Distinctive reverse side pigment								
	2	12	0	12	0	2	0	28
%	100	50	0	67	0	20	0	48
Spore bearing hyphae								
Biverticillium	2	18	0	16	4	8	0	48
%	100	75	0	89	100	80	0	83
Monovercicillium	0	6	0	2	0	2	0	10
%	0	25	0	11	0	20	0	17
Antimicrobial activity against								
<i>S. aureus</i>	0	12	0	8	0	6	0	26
%	0	50	0	44	0	60	0	45

Continued on p. 378

Table II continued

<i>E. coli</i>	0	2	0	2	0	0	0	4
%	0	8	0	11	0	0	0	7
<i>B. cereus</i>	0	10	0	8	0	6	0	24
%	0	42	0	44	0	60	0	41
<i>A. tumefaciens</i>	2	4	0	10	0	0	0	16
%	100	17	0	56	0	0	0	28
<i>P. aeruginosa</i>	2	12	0	18	0	8	0	40
%	100	50	0	100	0	80	0	69
Utilization of sugars								
Arabinose	2	22	0	18	4	10	0	56
%	100	92	0	100	100	100	0	97
Xylose	2	18	0	12	4	6	0	42
%	100	75	0	67	100	60	0	72
Inositol	0	20	0	8	1	6	0	35
%	0	83	0	44	25	60	0	60
Mannitol	2	22	0	16	4	8	0	52
%	100	92	0	89	100	80	0	90
Fructose	2	22	0	16	4	10	0	54
%	100	92	0	89	100	100	0	93
Rhamnose	2	20	0	14	4	10	0	50
%	100	83	0	78	100	100	0	86
Sucrose	2	10	0	12	4	10	0	38
%	100	42	0	67	100	100	0	66
Raffinose	0	6	0	2	4	0	0	12
%	0	25	0	11	100	0	0	21

Acknowledgement. The author wishes to thank the Dean of Academic Research and Higher Studies for funding this research proposal.

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ANTIBIOTIC RESISTANCE OF *ACINETOBACTER CALCOACETICUS* STRAINS ISOLATED FROM PATIENTS TREATED IN INTENSIVE CARE UNITS

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(Received January 13, 1995)

(Accepted February 10, 1995)

The distribution according to specimens and susceptibility to antimicrobial agents of 481 *Acinetobacter calcoaceticus* strains isolated from patients treated in intensive care units were studied. They occurred most frequently in tracheal specimens and pus. Using disk diffusion test the strains proved to be multiple resistant to ampicillin (86.3%), azlocillin (86.7%), mezlocillin (84.0%), cefamandole (99.7%), cefoxitin (94.1%), cefuroxime (90.6%), cefoperazone (84.9%), cefotaxime (82.0%), ceftriaxone (81.0%), tobramycin (71.2%), gentamicin (86.2%), chloramphenicol (90.5%) and tetracycline (89.8%). Based on the lowest incidence of resistant strains imipenem (0%), netilmicin (2.6%), amikacin (4.9%), ampicillin + sulbactam (8.9%), amoxicillin + clavulanic acid (29.4%), pefloxacin (26.2%), ciprofloxacin (30.1%), ofloxacin (34.3%), cotrimoxazole (41.6%), carbenicillin (41.2%) or ceftazidime (55.4%) may be the drug of choice in nosocomial *A. calcoaceticus* infections.

The *Acinetobacter* species are commonly present in different environmental samples and can be isolated from the skin, throat, conjunctiva and vagina of healthy individuals. During the last decade these bacteria have emerged as important nosocomial pathogens [1, 2]. Many colonizations and outbreaks of infections in patients at intensive care units with highly resistant *Acinetobacter* strains have been reported [3–7]. According to our experience in recent years, the species *Acinetobacter calcoaceticus* proved to be the most resistant bacteria to different antibiotics. The aim of this study was to evaluate the effectiveness of available antibiotics against *A. calcoaceticus* in the past three years.

Materials and methods

Strains. The 481 strains of *A. calcoaceticus* included in this study were isolated from different clinical bacteriological specimens obtained from the intensive care units of hospitals located in Hajdú-Bihar county in Hungary.

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Isolation and identification of strains were performed according to the methods recommended by the Hungarian National Institute of Hygiene [8, 9]. Isolates which met the following criteria were considered as *A. calcoaceticus*: negative or weak acid production in the upper part of a modified Russel type agar [8]. Acid production in the upper part of a glucose containing OF (oxidation/fermentation) agar. Negative nitrate reduction, utilization of ammonium citrate as a single carbon source, acid production from glucose and xylose, oxidase negativity, strong catalase reaction and no motility.

Antibiotic susceptibility tests were done by the disk diffusion method on Mueller–Hinton agar (Diagnostics Pasteur, France) [8], using ampicillin, 20 µg; carbenicillin, 100 µg; azlocillin, 30 µg; mezlocillin, 30 µg; cefamandole, 30 µg; cefoxitin, 30 µg; ceftriaxone, 30 µg; cefotaxime, 30 µg; gentamicin, 20 µg; tobramycin, 10 µg; amikacin, 30 µg; ofloxacin, 5 µg; chloramphenicol, 30 µg; tetracycline, 30 µg; trimethoprim + sulfamethoxazole, 25 µg (Resistest disks, Human, Budapest, Hungary), amoxicillin + clavulanic-acid, 20 µg; ampicillin + sulbactam, 20 µg; cefuroxime, 30 µg; cefoperazone, 30 µg; ceftazidime, 30 µg; ciprofloxacin, 5 µg; imipenem, 10 µg (OXOID Unipath Limited, Basingstoke Hampshire, U.K.); pefloxacin, 5 µg (Sanofi-Diagnostics Pasteur, France), and netilmicin, 30 µg (MAST Laboratories Ltd, Liverpool, England). In selecting the antibiotics for testing the NCCLS [10] recommendations were considered.

Results

The 481 isolates of *Acinetobacter* showed uniform biochemical activities thus they could be classified as *Acinetobacter calcoaceticus*. Their distribution according to clinical specimens is shown in Table I. They occurred at a highest rate in tracheal specimens and pus.

The results of the susceptibility testing to beta-lactam antibiotics (Table II) indicate that more than 80% of the isolates were resistant against all beta-lactams tested except carbenicillin and ceftazidime. In addition, a substantial portion of the strains was moderately sensitive to these two beta-lactams, too.

There was a considerable and interesting difference in the effectiveness of the two preparations containing inhibitors for beta-lactamases. Clavulanic acid seemed to exert a partial inhibition on penicillinases resulting in a high rate of moderately susceptible strains to the combination of amoxicillin and clavulanic acid when a good number of isolates was examined. In contrast, sulbactam had a strong inhibitory effect on beta-lactamases rendering most of the ampicillin-resistant strains susceptible to the combination of ampicillin and sulbactam in the case of a relatively small number of isolates tested. Imipenem examined for 29 strains proved to be effective in 100% (data not shown).

Of the aminoglycosides (Table III), the commonly used gentamicin and tobramycin showed a high rate of ineffectiveness exhibiting 86.2% and 71.2% resistant strains, respectively. In the contrary, netilmicin proved to be considerably effective with 34.3% susceptible strains and 63.1% moderately susceptible ones, while amikacin displayed the strongest effect with 67.5% susceptible isolates and 27.6% intermediate susceptibility.

Table I

Percentage distribution of A. calcoaceticus isolated from intensive care units

Specimen	Year							
	1991		1992		1993		Total	
	No. of strains	%	No. of strains	%	No. of strains	%	No. of strains	%
Ear swab	1	0.9	1	0.5	4	2.6	6	1.2
Nasal swab	1	0.9	—	—	—	—	1	0.2
Pharyngeal swab	5	0.9	3	1.4	6	4.1	14	2.9
Sputum	11	9.6	18	8.1	13	9.0	42	8.7
Tracheal sample	38	33.0	83	37.6	53	36.6	174	36.2
Pus	28	24.3	77	34.8	58	40.0	163	33.9
Wound	4	3.5	4	1.8	—	—	8	1.7
Bile	—	—	1	0.5	—	—	1	0.2
Urine	5	4.3	15	6.8	2	1.4	22	4.6
Cerebrospinal fluid	—	—	1	0.5	1	0.7	2	0.4
Blood culture	12	10.4	13	5.9	2	1.4	27	5.6
Others	10	8.7	5	2.3	6	4.1	21	4.4
Total	117	100	221	100	145	100	481	100

Fluoroquinolones showed only relatively good effects on *A. calcoaceticus* strains (Table IV) since the resistance rates ranged from 26.2% to 34.3%. Of the traditional and widely used antimicrobial agents, chloramphenicol and tetracycline proved to be practically ineffective with a resistance rate of 90%. It is worth to mention that cotrimoxazole showed a relatively considerable effect since "only" 41.6% of the strains exhibited resistance to this combination.

On the whole, strains of *A. calcoaceticus* isolated from intensive care patients proved to be multiple resistant to beta-lactams, aminoglycosides, chloramphenicol and tetracycline. Based antibiotic susceptibility testing and on the lowest incidence of resistant strains, imipenem, netilmicin, amikacin, ampicillin + sulbactam, amoxicillin + clavulanic acid, the fluoroquinolones, cotrimoxazole, as well as carbenicillin or ceftazidime may be the drug of choice in nosocomial *A. calcoaceticus* infections confirmed by.

Table II

Resistance pattern of A. calcoaceticus strains against beta-lactam antibiotics

Beta-lactam antibiotics	No. of strains examined	Percentage		
		Susceptible	Moderately susceptible	Resistant
Ampicillin	380	6.3	7.4	86.3
Azlocillin	421	5.7	7.6	86.7
Mezlocillin	432	4.4	11.6	84.0
Carbenicillin	444	27.5	31.3	41.2
Amoxicillin + clavulanic acid	350	13.7	56.9	29.4
Ampicillin + sulbactam	56	82.1	9.0	8.9
Cefamandole	317	0.3	—	99.7
Cefoxitin	405	3.2	2.7	94.1
Cefuroxime	350	2.9	6.5	90.6
Cefoperazone	205	5.3	9.8	84.9
Cefotaxime	296	4.0	14.0	82.0
Ceftriaxone	337	3.9	15.1	81.0
Ceftazidime	287	16.0	28.6	55.4

Discussion

The occurrence of *A. calcoaceticus* seems to be common in intensive care units and the infections are almost always present [11]. It has been detected in infections of patients with neoplasm disorders [12], burn injuries [13] and in complications following neurosurgical interventions [14]. It has also been occurred as pathogenic agent of pneumonia [15], lung abscess, septicaemia, various wound infections [2] and catheter related infections [16]. Beside these it can be a member of the normal skin flora of healthy persons [17]. It can also be cultured from the hospital environment such as humidifiers, airconditioners [18], liquid soaps [19] and even from the hands of nursing staff [1].

Thus, *A. calcoaceticus* plays an important role among the pathogens causing problems in the antibiotic therapy of nosocomial infections e.g.: methicillin resistant staphylococci and *Pseudomonas aeruginosa*. The greatest problem is the multiple resistance of these strains to different antibiotics [20].

Table III

Frequency of resistance of A. calcoaceticus strains against aminoglycosides

Aminoglycoside	No. of strains examined	Percentage		
		Susceptible	Moderately susceptible	Resistant
Tobramycin	427	12.4	16.4	71.2
Gentamicin	449	10.9	2.9	86.2
Amikacin	305	67.5	27.6	4.9
Netilmicin	428	34.3	63.1	2.6

Table IV

Effect of three types of fluoroquinolones and three traditional widely used drugs on A. calcoaceticus strains

Antimicrobial agent	No. of strains examined	Percentage		
		Susceptible	Moderately susceptible	Resistant
Pefloxacin	107	36.4	37.4	26.2
Ciprofloxacin	438	33.1	36.8	30.1
Ofloxacin	282	24.7	41.0	34.3
Chloramphenicol	431	3.7	5.8	90.5
Tetracycline	401	6.0	4.2	89.8
Trimethoprim + sulfamethoxazole	449	16.9	41.5	41.6

High resistance against beta-lactam antibiotics is generally attributed to beta-lactamase production [3, 21]. The elaboration of this enzyme, however, may not be the only cause of the beta-lactam multiresistance. The extremely low porin contents of *Acinetobacter* cell membranes may also play a role in it [5]. The relatively low incidence of resistance to carbenicillin and ceftazidime has previously been reported from other fields, too [11] and can be attributed to a variety of molecular mechanisms [21].

The favourable results with ampicillin + sulbactam, in contrast to amoxicillin + clavulanic-acid, indicate sulbactam to be a stronger inhibitor than clavulanic acid

rendering ampicillin-resistant strains of *Acinetobacter* susceptible to ampicillin + sulbactam. Our results are in accordance with earlier findings in Hungary [22].

There was a marked difference in the resistance rates to aminoglycosides. Most of the strains proved to be resistant to the older and commonly used tobramycin and gentamicin of which the mechanisms of resistance have been well defined [3, 21, 22], while they remained susceptible to netilmicin and amikacin. Amikacin- and ceftazidime-susceptible strains of *Acinetobacter* have also been reported from different areas of Liverpool [11] but amikacin was found not to be resultful in acinetobacter pneumonia and meningitis.

The considerably high incidence of resistant *A. calcoaceticus* strains to ofloxacin, ciprofloxacin and pefloxacin is alarming since especially ciprofloxacin should be a safety antimicrobial drug. Probably here again, as in case of staphylococci "A" type DNA gyrase is responsible for the appearance of resistance [21, 23, 24]. There are some data showing the development of resistance to ciprofloxacin during and after therapeutical intervention [23].

The indiscriminative use of antibiotics and the absence of an antibiotic policy might be responsible for the present situation in Hungary. Our results obtained by the disk diffusion method seem to be similar to the majority of other publications with some exceptions [15].

Because of the frequent presence of *Acinetobacter* strains in bacteriological samples from intensive care units there is a need for a clinical-epidemiological approach. Since in Hungary at present there is no approved routine mode of typing of *Acinetobacter* isolates except for experimental purposes [25], we suggest the introduction of one of the following typing methods at least: determination of cell membrane protein profiles [26], phage typing [27], serotyping [25, 28, 29] or bacteriocin-typing [30].

Acknowledgements. We would like to thank the employees of our bacteriological laboratory for their assistance, and Ms. Zoltáné Rákos for technical helps.

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IS SEMINAL FLUID A SUITABLE SPECIMEN FOR DETECTING CHLAMYDIAL INFECTION IN MEN?

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(Received January 31, 1995)

(Accepted February 21, 1995)

As genital chlamydia infection is considered the most prevalent sexually transmitted bacterial disease worldwide, its correct diagnosis has a great clinical and epidemiological importance. Urethra is the recommended site for collecting specimen for detection of *Chlamydia trachomatis* in men, but urethral swabbing is very inconvenient for the patients. Testing of seminal fluid has two advantages: taking the sample is more convenient for the patient, and the ejaculate would provide more information on the possible infection of the upper genital tract. Three non-culture methods – enzyme immunoassay (EIA), DNA hybridization, and polymerase chain reaction (PCR) – were used for evaluation of suitability of seminal fluid as a sample for detection of *Chlamydia* in 4 patient groups comprising 259 symptomatic and asymptomatic, urology and andrology patients. The seminal fluids were tested parallel with the urethral samples. In one group of patients with typical prostatic complaints expressed prostatic secretion was examined parallel with urethral swab. *C. trachomatis* positivity rates were much higher in symptomatic (49.1%) than in asymptomatic patients (12.3%) in all kinds of sample. Seminal fluid was somewhat more frequently positive (7.9%) than urethral sample (6.2%). Simultaneous positivity was relatively rare (3.3%), it occurred most frequently in samples of patients with possible accessory gland infection tested by EIA. In a group of 17 patients with typical prostatic complaints the expressed prostatic secretion alone was positive in 7 cases, whereas only other two of the parallel urethral samples proved positive by PCR, simultaneous positivity occurred in one case. These findings suggest that testing of the ejaculate and/or the prostatic secretion parallel with the urethral sample would improve the sensitivity of detection of chlamydial infections.

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Genital chlamydia infection is considered the most prevalent sexually transmitted bacterial disease worldwide [1–3]. In the United States more than 4 million chlamydial infections occur annually [2]. Most infected men and women are asymptomatic, and many of them transmit their infections to others [2, 4]. Although urethra is the recommended site for collecting specimens from men, obtaining urethral sample is associated with a discomfort for the patients. Moreover, the seminal fluid would provide more information on the possible infection of the upper genital tract. Recent developments in the laboratory diagnosis of chlamydial infections – enzyme immunoassay (EIA), DNA hybridization, and polymerase chain reaction (PCR) – offered new alternatives to compare the diagnostic value of testing urethral sample and seminal fluid.

Materials and methods

Patients examined and methods used are shown in Table I.

Urethral specimens were taken 1–2 hours after voiding, and handled according to the manufacturer's instructions. After urethral swabbing seminal fluid was taken (by masturbation after 5 days abstinence). Since there was no information on the preparation of the ejaculate in the original instructions for use, after leaving the seminal fluid to stand for 20–30 min, the swab supplied in the specimen collection kits was rotated in it for 5–10 s, and placed immediately into the transport medium. In group C the expressed prostatic secretion was examined instead of seminal fluid. After urethral sampling, prostatic massage was performed then a second urethral swab was taken. In the following both seminal and prostatic samples were handled according to the manufacturer's instructions. (In contrast to DNA and EIA methods, in case of PCR the swab was removed from the transport medium.)

In 50 cases of group A and in 18 cases of group D after centrifugation of the semen, in addition to the pellet, the supernatant was also examined parallel with the whole seminal fluid.

Results

Chlamydia trachomatis positivity rates were much higher in symptomatic patients (49.1%) than in asymptomatic ones (12.3%) in all kinds of sample (Tables II and III).

There was no close correlation between the positivity of seminal fluids and urethral samples: seminal fluids were somewhat more frequently positive than the latter ones (Table IV). Simultaneous positivity occurred rarely, it was most frequent in patient group D examined by EIA. In group C comprising patients with typical prostatic complaints, parallel with the urethral sample expressed prostatic secretion was tested by PCR (Table IV), and a much higher positivity rate was found in the latter samples (8/17 versus 3/17).

In case of DNA hybridization test (group A) out of 50 randomly selected samples 7 were positive by testing the whole seminal fluid, whereas all the corresponding pellets and supernatants were negative. After centrifugation the pellet and supernatant of 17 ejaculates were examined parallel with the whole semen by EIA technique (group D) as well: out of 8 positive whole seminal fluids all the corresponding supernatants, and all

but one pellets were positive. Both fractions of the negative whole samples retested after centrifugation proved negative.

Table I

Patients and methods

Patient group	Institution	Characterization of the group	Samples	No. of patients	Methods and kits used for detection <i>Chlamydia</i> infection
A	Andrology Centre, Department of Urology, Semmelweis University Medical School, Budapest	Asymptomatic, unexplained infertility	Urethral swab and seminal fluid	189	DNA hybridization: Gen-Probe ^R PACE ^R 2 System, Gen-Probe Inc., USA*
B	Urology Outpatient Clinic, Dabas	Asymptomatic, examined for possible urological focuses	Urethral swab and seminal fluid	15	
C	"Prostatitis" Outpatient Clinic, Department of Urology, Semmelweis University Medical School, Budapest	Symptomatic, typical prostatic complaints	Urethral swab and expressed prostatic secretion	17	PCR: Amplicor TM , Roche Diagnostic Systems, Switzerland**
D	Urology Outpatient Clinic, Dabas	Symptomatic, possible male accessory gland infection	Urethral swab and seminal fluid	38	EIA antigen detection: Chlamydia DEIA TM , Dako A/S, Denmark***

* Kindly provided by Epignost GmbH, Austria

** Kindly provided by Dako A/S, Denmark

*** Kindly provided by F. Hoffmann-La Roche AG, Hungary

Discussion

There are two advantages of testing seminal fluid for detection *Chlamydia* in men: (i) the sample collection is more convenient for the patient than urethral swabbing, (ii) ejaculate could be a possible specimen for diagnosis of the infection of the upper genital tract. Similarly to other authors [5-7], we have found elementary bodies of *C.*

trachomatis in the prostatic tissue by in situ hybridization test. In addition, elementary bodies were also detected in intracanalicularly located macrophages (unpublished data). Accordingly we have concluded that chlamydial elementary bodies can originate not only from the urethra but from the upper parts of the genital tract as well (i.e. prostatic gland). Consequently, ejaculate could provide more information on the possible infection of the male upper genital tract.

In the course of our earlier investigations seminal fluid samples were cultured in order to detect the presence of *Chlamydia* [4, 8]. However, cell culture isolation proved a technically difficult, time-consuming method not suitable for screening purposes. Moreover, several constituents of seminal fluid e.g. zinc, selenium and some peptides have an antichlamydial activity [1].

Table II

C. trachomatis positivity rate in asymptomatic patients

Method	No. of patients	No. of positive samples			
		Seminal fluid positive only, No.	Urethral sample positive only, No.	Both positive, No.	Total positive, No.
DNA hybridization	189 (group A)	11	9	2	22
PCR	15 (group B)	2	1	0	3
Total No.	204	13 (6.4%)	10 (4.9%)	2 (1.0%)	25 (12.3%)

Table III

C. trachomatis positivity rate in symptomatic patients

Method	No. of patients	No. of positive samples			
		Seminal fluid or EPS* positive only, No.	Urethral sample positive only, No.	Both positive, No.	Total positive, No.
EIA	38 (group D)	6	5	6	17
PCR	17 (group C)	7*	2	1	10
Total	55	13 (23.6%)	7 (12.7%)	7 (12.7%)	27

*Expressed prostatic secretion

Table IV

C. trachomatis positivity rate in seminal fluid and urethral sample by different methods

Method	No. of patients	No. of positive samples			
		Seminal fluid positive only, No.	Urethral sample positive only, No.	Both positive, No.	Total positive, No.
DNA hybridization	189 (group A)	11	9	2	22
EIA	38 (group D)	6	5	6	17
PCR	15 (group B)	2	1	0	3
Total	242	19 (7.9%)	15 (6.2%)	8 (3.3%)	42 (17.3%)

Our present results indicated that new non-culture techniques offered a means to evaluate the suitability of seminal fluid as a specimen for detection of chlamydial infections: ejaculate alone was more often positive than urethral swab by all methods used in every patient group. It can be assumed that the first site of infection is located in the urethra, and it may ascend to the male accessory glands, e.g. to the prostatic gland causing chronic, often asymptomatic infections. The positivity rates of seminal fluid and prostatic secretion samples found in andrology and urology patients suggest that the chlamydial infection of the male accessory glands may persist longer than that of the urethra.

Comparative evaluations of non-culture techniques for testing urethral swab have been published by several authors [2, 9–12], but there is only scanty information on using seminal fluid as a test sample [13]. Comparative examinations on the diagnostic value of urethral and seminal specimens have not been published. Therefore one should consider the reliability of the tests with semen. The question arises mainly in case of the DNA hybridization test, because the high somatic cell content of the seminal fluid may interfere with the reaction. Our controversial results obtained by testing the whole ejaculate and the corresponding pellet and supernatant suggest that there is a need for further investigations.

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INTERACTION BETWEEN *BORDETELLA PERTUSSIS* VACCINE AND CHLORPROMAZINE IN MOUSE EXPERIMENTS

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(Received February 21, 1995)

(Accepted March 23, 1995)

In acute toxicity experiments the mortality of mice pretreated with *Bordetella pertussis* vaccine increased on the first day following chlorpromazine (CPZ) treatment, compared to control animals treated with CPZ alone. Initially, the increased drug sensitivity observed after combined treatment was attributed to summation of the toxic effects. However, the cumulation of mortality did not cease on the following days, furthermore, an increase of bacterial translocation was observed on days 6 and 7, i.e. when the lymphocytosis, splenic hypertrophy and shrinkage of thymus – changes consequent to the vaccination – were at their maximum levels. On the basis of all these and on literary data it is supposed that the early cumulation of deaths after combined treatment may be in connection with an interaction between the two agents and that the side-effects following vaccination of humans may be induced by undesirable pharmacological interactions.

In our earlier experiments [1, 2] germ-free (Gf) mice proved to be more resistant to the lymphotropic cytostatic drug dianhydrotolcitol (DAD) than conventional (Cv) mice of similar age. In our further experiments [3] the calmodulin antagonist chlorpromazine (CPZ) induced an immunosuppressive effect, furthermore, Gf mice proved to be much less sensitive to CPZ than Cv mice.

We explained the higher drug sensitivity (DS) of Cv mice by translocation of bacteria from the normal enteric flora in the immunosuppressive condition into otherwise sterile visceral organs (bacterial translocation, BT) and a consequent endotoxin effect [4, 5].

We [6] and other investigators also found, that Mannozyne (M), a glycomannan preparation obtained from *Saccharomyces cerevisiae*, caused well-defined splenic hypertrophy and, at the same time, it proved to be an immunostimulant. In the state of M-induced splenic hypertrophy an increased DS could not be detected as a response to any of the following agents: DAD stereoisomer, DAD and CPZ; the BT rate was not influenced by M [7].

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We also proved [8] that *Bordetella pertussis* vaccine treatment was followed by an increase in sensitivity to DAD in both Gf and Cv mice. The immunomodulant effect of the vaccine was age-dependent: the cellular immune response to LCM virus infection was stimulated by the vaccine in suckling mice but it was suppressed in young adult and old ones [9–11]. The vaccine caused splenic hypertrophy, lymphocytosis, and shrinkage of the thymus, changes independent of the direction of immunomodulation [12].

In the present work adult Cv mice were treated with an immunosuppressive *B. pertussis* vaccine.

We attempted to answer the following questions:

- (i) does the sensitivity of young adult Cv mice to CPZ change ?
- (ii) is BT detectable in the state of splenic hypertrophy caused by the vaccine?
- (iii) is the rate of CPZ-induced BT influenced by the vaccine?

Materials and methods

Experimental animals. CD-1 (Charles River Co) mice of both sexes, 6 to 8 weeks of age.

Vaccine treatment. The *B. pertussis* vaccine was produced by HUMAN Co. Ltd, Budapest-Gödöllő. It contained 30×10^9 killed bacterial cells/ml. The mice were injected intraperitoneally (i.p.), each with 0.3 ml of vaccine (9×10^9 cells/mouse).

Chlorpromazine treatment. 2.5% solution of chlorpromazinum hydrochloricum was used. The further dilutions, necessary for the different doses, were prepared in PBS immediately before used in experiment. The mice were inoculated on one occasion each.

Examination of bacterial translocation. Preparation of cell suspension. After the mice were killed, two mesenterial lymph nodes, the spleen and the liver were removed with sterile instruments. Cell suspension was prepared in 0.5 ml PBS from each of the lymph nodes and from a 100 mg fragment of each spleen and liver.

Isolation of bacteria. The whole quantity of each of the cell suspensions was inoculated into serum broth medium. From the inoculated medium subcultures were made on blood agar in every 24th h.

The colonies grown on the latter were examined by macroscopic, microscopic and biochemical methods with help of the "ATB EXPRESSION AUTOMATIC MICROBIOLOGICAL IDENTIFICATION and ANTIBIOTIC SENSITIVITY SYSTEM". The test was considered negative when none of the subcultures made on each of the five days of incubation produced visible growth on the agar plates within 48 h of incubation.

Determination of translocation index

$$\text{translocation index: } \frac{\text{translocation percent in the treated group}}{\text{translocation percent in the control group}}$$

Examination of the lymphoid system

(a) Lymphocytes were counted in blood samples taken from the tail vein (ly count);

(b) ly index: $\frac{\text{average ly count for the treated group}}{\text{average ly count for the control group}}$

- (c) relative spleen weight: $\frac{\text{spleen weight mg}}{\text{body weight g}}$
- (d) spleen index: $\frac{\text{average relative spleen weight for the treated group}}{\text{average relative spleen weight for the control group}}$
- (e) relative thymus weight: $\frac{\text{thymus weight mg}}{\text{body weight g}}$
- (f) thymus index: $\frac{\text{average relative thymus weight for the treated group}}{\text{average relative thymus weight for the control group}}$

Statistical analysis. Student's *t*-test was applied. $P=0.05$ was accepted as limit of significance.

Experiments and results

Three experiments (Experiments I, II and III) were performed. The grouping of mice, the numbers of mice and the treatment of mice are shown in Table I. In experiment I, the effect of *B. pertussis* vaccine on the lymphatic organs is shown. The examinations were carried out on the 7th day following vaccine treatment. The vaccine treatment induced significant increase in the ly count, well-defined splenic hypertrophy and significantly decreased relative thymus indices (Table II).

Table I

Grouping and numbers of mice in treated in the experiments

Group	No. of mice in experiments			I.p. treatments, <i>B. pertussis</i> vaccine, 9×10^9 bacterial cells/per mouse	CPZ 75 mg/kg	PBS
	I	II	III			
P-CPZ	—	20	40	+	+	—
CPZ	—	20	40	—	+	—
P	20	20	20	+	—	—
C	20	20	20	—	—	+

P = *B. pertussis* vaccine, C = control

In Experiment II it was studied whether the time and the rate of CPZ mortality was influenced by *B. pertussis* vaccination. The mice were injected with 75 mg/kg CPZ on the first day following the vaccine treatment. Mortality was registered daily on the following 21 days. As shown in Fig. 1 *B. pertussis* vaccine in itself did not cause mortality at all. The CPZ dose in use alone and the same combined with the vaccine led to 20% and 93% mortality, respectively. In the P-CPZ group deaths cumulated on the first day following treatment while the animals were sleeping.

Table II

Effect of B. pertussis vaccine on the lymphatic organs of mice

Group	Ly count×1000	Ly index	Rel. spleen weight	Spleen index	Rel. thymus weight	Thymus index
P	133±73	2.5	14.1±2.7	4.7	2.8±0.5	0.6
C	5.3±1.4	1	3±0.8	1	4.6±1.1	1
P versus C	P<0.001		P<0.01		P<0.01	

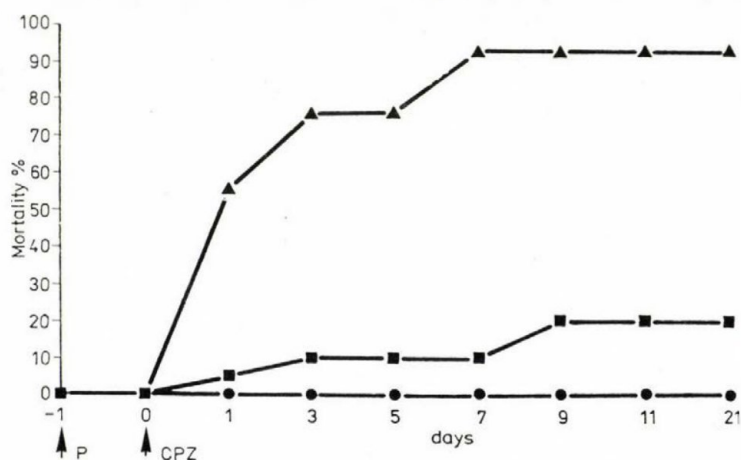


Fig. 1. Dynamics of mortality. Experiment I/A. Separate treatment with *B. pertussis* and CPZ, and combined treatment: ● P, ■ CPZ, ▲ P-CPZ

In Experiment III, the dynamics of BT was examined after separate and combined treatment. CPZ was administered on postvaccination day 1, whereas re-isolation was attempted on day 6.

By the 6th day mortality had reached 45% and 25% in group P-CPZ and group CPZ, respectively. The mice still living on day 6 were killed and isolation of bacteria was attempted from two peritoneal lymph nodes, the spleen and the liver of each of the killed mice (Table III).

Table III

Bacterial translocation rate for mice and for mouse organs tested in Experiment III

Groups of mice	Translocation rate				Translocation index	
	mice positive/total	%	organs positive/total	%	mice	organs
P-CPZ	10/22	45.4	10/66	15.1	4.5	4.5
CPZ	8/30	26.6	8/90	8.8	2.6	2.6
P	4/20	20.0	4/60	6.6	2.0	2.0
C	2/20	10.0	2/60	3.3	1	1

In Table III the BT rates for mice and those calculated for their organs are presented. The BT rate was the highest in the group given combined treatment: it was 45% for the mice and 16.7% for their organs. Similarly to the BT rate, the translocation indices were the highest in the P-CPZ group. In accordance with our earlier experiment [6] moderately increased BT rates (26.6% and 8.9%, respectively) were obtained on days 6 and 7 following treatment with 75 mg/kg CPZ. As compared to group C (control) the increment in BT was the lowest in the group given pertussis vaccine alone (group P).

Twenty-four bacterial strains were isolated from the mice altogether: 18 from lymph nodes, two from spleens and four from livers. In the CPZ-treated groups both Gram-positive (*Staphylococcus aureus* and *Streptococcus faecalis*) and Gram-negative bacteria (*Escherichia coli*, *Proteus*) were found, whereas from the control groups and in the groups pretreated with the vaccine alone only Gram-positive strains (*S. faecalis*) were isolated.

Discussion

Similarly to our earlier experiments, in Experiment I of the present work we detected lymphocytosis, splenic hypertrophy and shrinking thymus in mice pretreated

with *B. pertussis* vaccine. These changes can be attributed to multiple effects of the vaccine displayed on the lymphatic system (13).

In Experiment II, mice that had undergone combined treatment with pertussis vaccine and CPZ – both being immunosuppressive agents – responded with a considerably increased DS to combined treatment as indicated by cumulation of deaths after treatment. On the other hand, in our earlier experiments [7], in which CPZ treatment was combined with Mannozy (zymosan), another agent causing splenic hypertrophy but being an immunostimulant, we failed to induce similar increase in DS.

In Experiment II cumulation of deaths was observed in group P-CPZ on the first day following CPZ treatment; most of the deaths occurred while the mice were sleeping. In earlier studies an increased BT was demonstrated after treatment with immunosuppressive agents (DAG and CPZ); the BT curve reached its peak on postinjection days 3 to 6 [14]. Initially, we attributed the increased DS of Cv mice, compared to that of Gf mice, to a possible BT induced by the immunosuppressant (CPZ, DAG) [14]. Increased DS was observed in the present experiment as well, in mice treated with a P+CPZ combination, however, there is no doubt that BT cannot cause cumulation of deaths as soon as on the first postinjection day. Thus, one should look for other (co)factors to explain the increased DS. This conclusion is in accordance with our earlier findings, namely that the increased DS was found by us in mice that had received combined treatment with *B. pertussis* vaccine and DAG. In the experiments referred to, even Gf mice responded with an increased DS. Of course, in Gf mice, in which the normal intestinal flora is lacking, BT as DS-increasing factor cannot be taken into consideration.

In Experiment II, the distance between the mortality curves CPZ and P-CPZ is increasing, but moderately, after the cumulation of deaths on day 1; daily mortality was consistently higher in the group with combined treatment than in the singly-treated groups. We observed an increased BT (Exp. III), compared to control values calculated for mice with CPZ treatment or for the nonfatal treatment with pertussis vaccine, however, the increase in these groups did not reach the rate calculated for the group treated with both agents. The increased BT for these groups can be explained by immunosuppression induced by separate or combined administration of the two agents.

The explanation of the early cumulation of deaths following combined treatment with the two agents used by us needs further research. An interaction may exist between CPZ and the *B. pertussis* vaccine, which manifests itself not only in the increased DS, but in addition it may potentiate the effects both of the vaccine, and CPZ.

Based on literary data we suppose that under experimental conditions both the pertussis toxin present in the whole-cell vaccine [15] as well as the CPZ [16, 17] affect the Ca mechanism of eukaryotic cells; furthermore the whole cell vaccine may induce side-effect of unknown mechanism in the human body [18]. Our experimental results and literary data suggest that the early cumulation of mortality may be connected with an interaction between the injected agents in both humans and experimental animals.

Acknowledgement. The present work was supported by the Hungarian Scientific Research Fund (OTKA) grant I/3 2612 "A".

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A HIGHER PRODUCTION OF PLATELET ACTIVATING FACTOR IN EX VIVO HETEROLOGOUSLY SECONDARY DENGUE-2 VIRUS INFECTIONS

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(Received March 20, 1995)

(Accepted April 14, 1995)

Patients with a dengue-1 virus (DV-1) infection followed by another DV-2 infection were reported to have a higher incidence of dengue haemorrhagic fever (DHF). Mononuclear leukocytes (MNLs) are principal cells for DV replication so that we challenge MNLs obtained from non-immune and previously DV-1-infected donors with DV-2 to mimic primary and heterologously secondary DV-2 infections. Production of platelet activating factor (PAF), thromboxane B₂ (TxB₂), and prostaglandin D₂ (PGD₂) by MNLs in the DV-2 infections was measured by radioimmunoassay. We found that MNLs, no matter primary or heterologously secondary exposure to DV-2, could release significant amount of PAF, TxB₂, and PGD₂. PAF but not TxB₂ or PGD₂ levels released by MNLs were significantly higher in those obtained from previously DV-1-infected donors (75.8 ± 28.5 vs 21.2 ± 13.4 pg/ml; $p < 0.05$). These results suggest that PAF, which is known to enhance inflammatory reactions and to augment platelet aggregation, may in part participate in the pathogenesis of DHF.

Primary dengue fever is a benign viral illness. Secondary dengue virus (DV) infections with different serotypes of dengue viruses are more frequently associated with serious complications such as dengue haemorrhagic fever (DHF), which tends to have a higher mortality [1, 2]. The risk of sequential DV infections, and incidence of DHF has increased dramatically first in Southeast Asia and later in the Americas and China [3-5]. Recently, it has been virologically confirmed that patients with primary dengue fever, no matter imported or indigenous, have emerged all over the world including French Polynesia [6], Sweden [7], the United States [3, 8], and Taiwan [9, 10]. Taken together, these results imply that dengue fever with DHF may outbreak in these areas if other serotypes of DV are transmitted into these areas. Pathogenesis of DHF is not completely understood. Patients with DHF usually present bleeding tendency, thrombocytopenia, disseminated intravascular coagulopathy, haemoconcentration, and even hypovolemic shock. Mononuclear leukocytes (MNLs) are the principal cells for DV replication [1, 2]. MNLs are known to secrete many proinflammatory cytokines and lipid mediators, which are implicated in many infection-mediated septic shock and vascular leakage syndromes

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[11, 12]. In an ex vivo model, we have recently shown that IL-11 β and TNF α released by MNLs with primary and heterologously secondary DV-2 infections were not significantly different [13]. We doubt if platelet activating factors such as platelet activating factor (PAF), thromboxanes, and prostaglandins are involved in the pathogenesis of DHF. In this study we challenged MNLs obtained from normal (non-immune) and previously DV-1-infected donors with DV-2 to mimic primary and heterologously secondary DV-2 infections and assessed production of platelet activating factors by MNLs with DV-2 infections.

Materials and methods

Preparation of mononuclear leukocytes. Mononuclear leukocytes (MNLs) were prepared from peripheral blood drawn from 8 non-immune donors living in northern Taiwan and 8 previously DV-1-infected patients who lived in southern Taiwan and were virologically confirmed to have DV-1 infections during 1988–1989 in our previous study [9]. Informed consents were completed before blood was drawn. Dextran (4.5%; T500, Pharmacia Fine Chemicals, Piscataway, NJ) was mixed with the peripheral blood at a ratio of 1:4 and the syringe was incubated nozzle upward at 37 °C for 30 min. Leukocyte-enriched plasma was harvested and separated into polymorphonuclear leukocytes and mononuclear leukocytes (MNLs) by a gradient centrifugation in Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ) at 1 500 *g* for 15 min [14]. After centrifugation, interface MNLs containing >98% viability and purity were collected, washed, and suspended at 2×10^6 cells/ml for studies.

Virus and virus titre. Dengue 2 virus (DV-2, New Guinea C strain) obtained from the Institute of Preventive Medicine, Taipei, Taiwan was utilized in this study. Viruses were propagated in the *Aedes albopictus* C6-36 cells [13, 15]. Virus titers were determined by a plaque-forming assay using BHK-21 cells as described previously [15, 16]. Viruses were pooled and concentrated to 2×10^7 plaque-forming units (PFU)/ml in RPMI 1640 medium, and stored at –70 °C before use.

Study design and measurement of inflammatory mediators. MNLs (2×10^6 cells/ml) obtained from non-immune and previously DV-1 infected donors were incubated in 24-well culture plates. Experiments were run in a paired control-experimental design and each experiment had duplicate reactions. MNLs obtained from non-immune donors were challenged with DV-2 at 1:1 MOI (multiplicity of infection) to mimic primary DV-2 infections; in contrast, MNLs from the donors with previous DV-1 infections between 1987 and 1989 [9] were challenged with DV-2 at 1:1 MOI to mimic heterologously secondary DV-2 infections. Both groups of MNLs were also incubated with medium as negative controls. Culture supernatants were harvested at 16 h after incubation. These samples were aliquoted and stored in –70 °C until radioimmunoassay (RIA) kits were available. PAF, TxB2, and PGD2 levels in the reaction supernatants were determined by RIA (Amersham's scintillation proximity assay, Amersham International plc, Amersham, UK).

Results

MNLs obtained from non-immune donors (non-immune MNLs) or from previously DV-1 -infected donors (DV-1-infected MNLs) cultured with medium alone did not release significant amount of PAF, TxB2, or PGD2. Non-immune MNLs as well as DV-1-infected MNLs in response to DV-2 for 16 h released significantly amount of PAF, TxB2, and PGD2, as shown in Table I. TxB2 levels released by non-immune

MNLs with primary exposure to DV-2 were not significantly different from those released by DV-1-infected MNLs with heterologously secondary exposure to DV-2 (232.1 ± 22.1 vs 281.5 ± 31.1 pg/ml; $p > 0.05$, t test, $n=8$). Similarly, PGD2 levels released from both groups of MNLs were not significantly different (17.6 ± 3.8 vs 20.1 ± 9.2 pg/ml; $p > 0.05$, t test). In contrast, PAF levels released by DV-1-infected MNLs with heterologously secondary exposure to DV-2 were significantly higher than those released by non-immune MNLs with primary exposure to DV-2 (Table I).

Table I

Production of PAF, TxB2, and PGD2 by MNLs with primary and heterologously secondary exposure to DV-2

Reactions	PAF (pg/ml)	TxB2 (pg/ml)	PGD2 (pg/ml)
Non-immune MNLs			
+ RPMI	undetectable	51.2 ± 8.5	6.9 ± 6.0
+ DV-2	$21.2 \pm 13.4^*$	232.1 ± 22.1	17.6 ± 3.8
DV-1-infected MNLs			
+ RPMI	undetectable	63.2 ± 11.3	10.0 ± 4.2
+ DV-2	$75.8 \pm 18.5^*$	281.5 ± 31.1	20.1 ± 9.2

Notes. MNLs (mononuclear leukocytes, 2×10^6 cells/ml) obtained from non-immune as well as previously DV-1-infected donors were challenged with and without DV-2 for 16 h. Levels of PAF (platelet activating factor), TxB2 (thromboxane B2), and PGD2 (prostaglandin D2) in the reaction supernatants were determined by radioimmunoassays. RPMI = RPMI 1640 medium, DV-2 = dengue 2 virus (2×10^6 PFU/ml). Data presented are mean $\pm$ SD ($n=8$). * Indicates a significant difference ($p < 0.05$, t test)

Discussion

Dengue fever and its severe form complication, DHF, have emerged as the most important arthropod-borne viral disease in Southeast Asia and South America [1-5]. People from Europe or North America who travel to tropical countries may also have an opportunity to get this disease [7, 8]. Evidence has shown that dengue fever appeared to be a benign disease before second World War [4]. However, multiple dengue serotypes may be geographically carried by viraemic troops, refugees, tourists or businessmen, and vectors including ova, larvae, and female mosquitos of *Aedes aegypti* may be carried by air or ship cargoes during and after World War II. The dissemination of viruses and

vectors may have promoted frequency of sequentially heterotypic DV infections, resulting in higher incidence of DHF [4]. Patients with DHF have been reported from Southeast Asia since 1950s [1, 3], from South America since 1970s [4, 8, 17], and from China since 1980s [5]. Interestingly, data from those epidemiological studies all demonstrated that outbreaks of several serotypes of endemic dengue fever usually occurred several years before a serious outbreak of DHF [1–5]. In a recent survey, there have been a number of cases with classic dengue fever found in Taiwan since 1987 [9, 10]. Although most of the cases had DV-1 infections, 3 other serotypes of DV were also sporadically identified [10]. We are very concerned about the possibility of an outbreak of DHF, though there has not been any patient with DHF identified in Taiwan since 1987 [9, 10]. Since patients with DHF tended to have a high mortality, and a high dose of steroid did not reduce mortality [18]. In this study, we sought to investigate if platelet aggregating and relaxing factors such as PAF, TxB₂, and PGD₂ were released by MNLs in in vitro primary and secondary DV-2 infections. We first identified that MNLs in response to DV-2 could release PAF, TxB₂, and PGD₂. Furthermore, we found that DV-1-infected MNLs in heterologously secondary exposure to DV-2 released significantly higher PAF levels than non-immune MNLs with primary DV-2 exposure.

Classically, circulating immune complexes had been implicated in the pathogenesis of dengue haemorrhagic fever and dengue shock syndrome in which immune complex-induced complement activation might cause disturbance of coagulation and vascular permeability [19]. Immune complexes as assessed by quantitative methods have been shown to be not the major factor in the complement activation of dengue fever [20]. In this study, we found that platelet aggregating and relaxing factors may be involved in the pathogenesis of dengue fever, and PAF may contribute to the higher incidence of DHF in secondary heterotypic DV infections. To testify this hypothesis in in vivo, further studies to monitor seral PAF levels in patients with classic dengue fever and DHF are needed in the future. If this hypothesis is proved, PAF antagonist may have a therapeutic potential for patients with DHF before a safe dengue virus vaccine is successfully developed.

Acknowledgement. This work was in part supported by grants NSC 81-0412-B016-503 and NSC 83-0412-B016-065 from the National Science Council, Taiwan, R. O. C.

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RADIODETOXIFIED ENDOTOXIN-INDUCED TOLERANCE. EFFECT ON ENDOTOXIN LETHALITY AND MACROPHAGE ARACHIDONIC ACID METABOLISM

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(Received April 10, 1995)

(Accepted May 16, 1995)

Endotoxin (LPS) tolerance produces changes in macrophage mediator production which is thought to be responsible for the acquired LPS resistance. Detoxification of LPS by gamma irradiation has been reported to diminish certain noxious properties while retaining its tolerance inducing actions. We compared the efficacy of LPS and radiodetoxified (RD)-LPS from *Escherichia coli* O101 on stimulating rat peritoneal macrophage arachidonic acid (AA) metabolism, measured by thromboxane (TXB₂). Changes in macrophage production of these mediators were also assessed after tolerance induction. LPS tolerance was induced by i.p. injection of LPS, RD-LPS or vehicle on day 1 (100 µg/kg, i.p.) and day 2 (500 µg/kg, i.p.). On day 5 or 4 weeks after pretreatment, peritoneal macrophages were harvested for in vitro studies, or rats were tested for lethality resistance. Macrophages were incubated ±LPS (0.1 ng to 50 µg/ml), lipid A (1 or 10 µg/ml) or Ca⁺⁺ ionophore A23187 (10 µM) for determination of TXB₂ production. Minimum effective concentrations of LPS and RD-LPS for stimulation (P<0.05) of TXB₂ were 100 ng/ml and 1 µg/ml, respectively. Maximal stimulation of TXB₂ occurred at 10 µg/ml of LPS or RD-LPS. Macrophages from LPS or RD-LPS tolerized rats were refractory to stimulated TXB₂ with LPS or RD-LPS (0.1 ng to 50 µg/ml). The suppressed in vitro macrophage TXB₂ production was apparent 4 weeks after rats were tolerized with LPS or RD-LPS. In subsequent mortality studies, LPS challenge of control or tolerance rats at day 5 in vivo with *Salmonella enteritidis* LPS (15 mg/kg, i.v.) produced a 90% mortality in control rats (N=22), versus 13% mortality in the LPS pretreated group (N=23) and a 20% in the RD-LPS pretreated group (N=10) (P<0.05 vs control). However, this lethality resistance was not apparent at 4 weeks after LPS or RD-LPS pretreatment. Both LPS and RD-LPS appear to be equipotent in inducing macrophage alterations, and in lethality resistance during LPS tolerance induction. However, these observations suggest that during LPS tolerance suppression of LPS-stimulated AA in peritoneal macrophage metabolism persists longer than acquired lethality resistance.

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The major cause of the sepsis syndrome is bacterial lipopolysaccharide (LPS), a cell wall component of Gram-negative microorganisms. The lethality of sepsis is not due to the toxicity of the LPS molecule per se, but to its effect on stimulating inflammatory cells, especially macrophages [1]. In response to LPS, macrophages produce a plethora of mediators which may effect the lethal sequelae of sepsis. The latter include arachidonic acid (AA) metabolites [2].

Sublethal administration of LPS in humans and laboratory animals diminishes macrophage release of certain inflammatory mediators, including arachidonic acid (AA) metabolites and produces resistance to lethal shock upon secondary challenge with LPS [3–5]. This acquired resistance to LPS is referred to as LPS tolerance. Hyporesponsiveness of macrophages to LPS is thought to be one factor in the development of LPS tolerance.

The phenomenon of LPS tolerance has prompted attempts to detoxify LPS to dissociate its toxicity from its tolerizing effects. One detoxification procedure is radiation treatment of LPS from *Escherichia coli* O101 [6, 7]. Several noxious properties of radiodetoxified (RD) LPS are diminished whereas the LPS tolerance-inducing effects of RD-LPS are preserved. The present study compared the effect of RD-LPS to native LPS in inducing LPS tolerance as measured by in vitro peritoneal macrophage TXB₂ production and resistance to LPS lethality.

Materials and methods

Tolerance induction and mortality studies. All rats used in these studies were pathogen free and viral antibody free (VAF) Long Evans male rats (180–200 g). Tolerance was induced by intraperitoneal injection of *E. coli* O101 LPS (Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary) for two consecutive days at 100 and 500 µg/kg body weight. Controls received isovolumetric doses of the sterile 5% dextrose vehicle. Experiments were performed at five days or four weeks after the first injection. On each study day, rats were used for in vivo mortality, or peritoneal cells were harvested for in vitro studies. *S. enteritidis* LPS (Difco Laboratories) was administered under light ether anesthesia intravenously (15 mg/kg) for mortality, and survival was monitored for 72 h.

Cell cultures. Peritoneal cells from LPS pretreated or control rats were harvested by peritoneal lavage following ether anesthesia at 5 days or four weeks after pretreatment. Cells in RPMI 1640 with L-glutamine medium containing penicillin (50 units/ml) and streptomycin (50 µg/ml) were plated at 5×10^5 cells/ml in flat-bottom 24-well plates and incubated at 37 °C and 5% CO₂. After 2 h non-adherent cells were removed by washing three times.

Macrophages were incubated for 3 h in 0.5 ml of RPMI media with antibiotics with or without *S. enteritidis* LPS, purified monophosphoryl lipid A from *S. minnesota* LPS, or media alone. Media were removed for TXB₂ radioimmunoassay.

TXB₂ radioimmunoassay. TXB₂ was quantitated by radioimmunoassay as previously described [8].

Data analysis. Statistical analyses were determined using Analysis of Variance (ANOVA) with intercomparison analysis being made using Fisher's Least Significant difference. Mortality studies were examined by the Chi-Square Test. A value of $P < 0.05$ was considered significant.

Results

The effect of *E. coli* O101 LPS or *E. coli* O101 RD-LPS on in vitro stimulation of TXB₂ was determined by peritoneal macrophages. RD-LPS significantly stimulated TXB₂ (threshold 100 ng/ml; $P < 0.05$) relative to LPS (threshold 1 $\mu\text{g/ml}$) (Fig. 1a). Although the degree of LPS stimulation did not vary between RD-LPS and LPS both LPS preparation appeared to produce optimal stimulation at (10 $\mu\text{g/ml}$). In subsequent studies the effect of in vivo pretreatment with the five day tolerance regimen of LPS or RD-LPS in vitro stimulation of peritoneal macrophage TXB₂ production with the respective LPS or RD-LPS was examined (Fig. 1b). Tolerance induced by either LPS preparation suppressed macrophage both basal and simulated TXB₂ production when tested in vitro with LPS or RD-LPS (0.1 ng to 100 $\mu\text{g/ml}$).

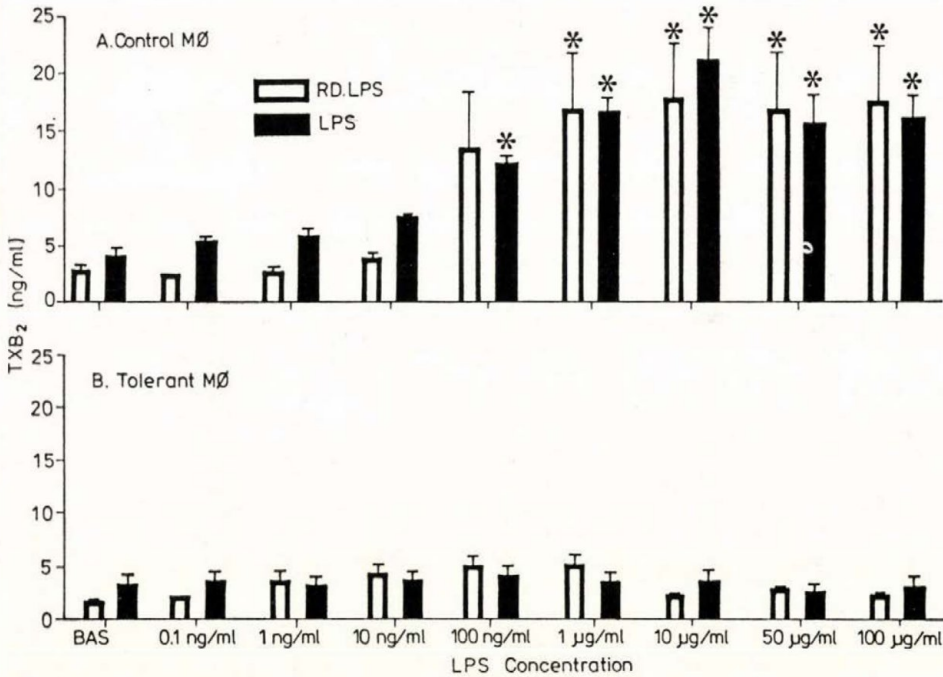


Fig. 1. (A) TXB₂ production in peritoneal macrophages stimulated in vitro with RD-LPS or LPS. In vitro stimulation of peritoneal macrophages TXB₂ without (BAS) and with LPS or RD-LPS cells were stimulated for 3 h. (B) In vitro stimulation without (BAS) and with LPS or RD-LPS on day 5 after tolerance induction with LPS or RD-LPS. * $P < 0.05$ versus Basal TXB₂

The effect of LPS or RD-LPS tolerance on in vitro TXB₂ production in response to several different in vitro agonist of macrophage arachidonic acid metabolism is compared (Figs 2, 3 and 4). In vitro stimulation with *S. enteritidis* LPS (50 µg/ml), calcium ionophore A23187 (10 µM), *S. minnesota* monophosphoryl lipid A (1 µg and 10 µg/ml) stimulated TXB₂ peritoneal macrophages from five day vehicle control rats (Fig. 2). In contrast, macrophages from LPS tolerant rats exhibited markedly suppressed responses to *S. enteritidis* LPS, and lipid A (1 µg and 10 µg/ml) compared to respective control groups of the five day vehicle pretreated group. The five day tolerant macrophages however did respond to A23187 although the response was reduced ($P < 0.05$) compared to its respective vehicle control. Similar results were achieved with the five day tolerant rats treated with RD-LPS (Fig. 3). All agonist stimulated TXB₂ production in vehicle controls. The five day RD-LPS treated group exhibited significantly suppressed TXB₂ production of each of the agonist relative to the respective vehicle control group ($P < 0.05$). The *S. enteritidis* LPS and *S. minnesota* LPS stimulated an attenuated increase of TXB₂ in the RD-LPS tolerant group compared to the intragroup basal TXB₂ production.

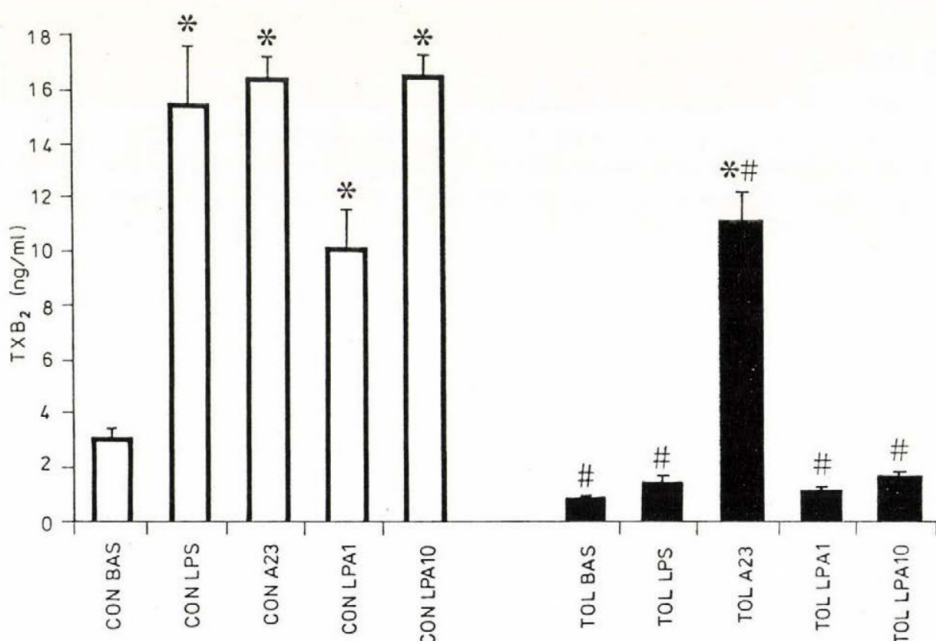


Fig. 2. Stimulated synthesis in macrophages on day 5 after reduction of tolerance with LPS TXB₂ levels were measured in media from control (CON) and tolerant (TOL) macrophages at 3 h after LPS (50 µg/ml), calcium ionophore A23187 (A23), (10 M), lipid A (LPA) (1 µg/ml and 10 µg/ml). Data are expressed as mean $\pm$ S.E.M. N=3 separate experiments. * $P < 0.05$ versus basal; # $P < 0.05$ versus respective CON group

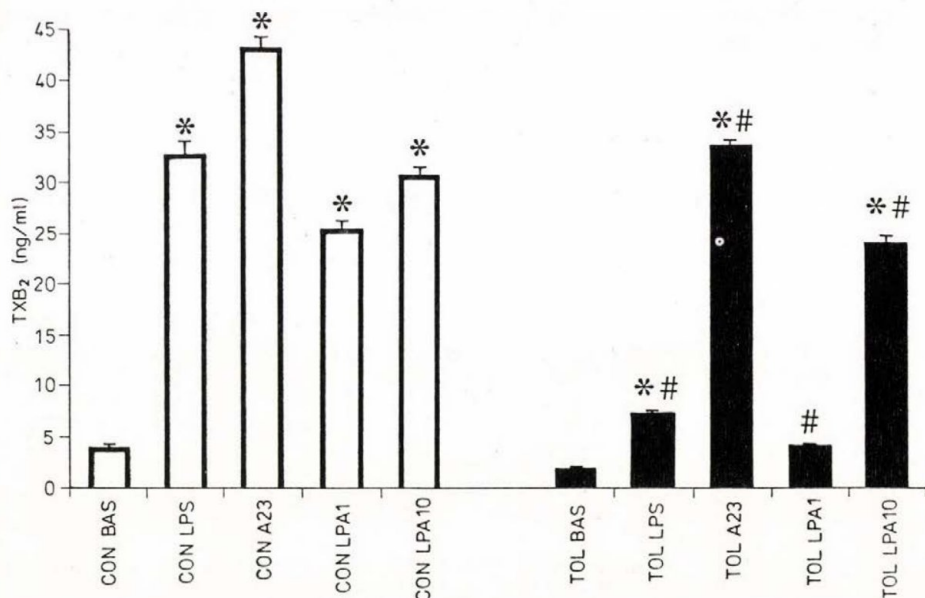


Fig. 3. Stimulated synthesis in macrophages on day 5 after reduction of tolerance with RD-LPS. TXB₂ levels were measured in media from control (CON) and tolerant (TOL) macrophages at 3 h after LPS (50 µg/ml), calcium ionophore A23187 (A23), (10 µM), lipid A (LPA) (1 µg/ml and 10 µg/ml). Data are expressed as mean ± S.E.M. N=3 separate experiments. *P=0.05 versus basal; #P=0.05 versus respective CON group

In subsequent studies, the effect of LPS or RD-LPS tolerance induction on in vitro macrophage TXB₂ production was determined at 4 weeks after tolerance induction (Figs 4 and 5). All in vitro agonist stimulated TXB₂ production in both the 4 week LPS vehicle and RD-LPS vehicle group ($P<0.05$) versus basal levels. However in LPS or RD-LPS tolerant groups agonist-reduced TXB₂ synthesis ($P<0.05$) were suppressed versus the respective vehicle treated groups. However unlike the five day LPS group, the cells appeared to exhibit recovery to the lipid A (10 µg/ml) stimulation versus basal even though the levels are still suppressed relative to the respective vehicle control (Fig. 3). The data observed with the 4 week RD-LPS TOLERANCE groups were qualitatively similar to the five day RD-LPS (Fig. 4).

To compare the changes in peritoneal macrophage mediator production to change changes in sensitivity to LPS, lethality studies were undertaken. Rats were given the 5 day or 4 week tolerance regimen of vehicle, LPS or RD-LPS (Fig. 6). On day five or at four weeks the rats were challenged with *S. enteritidis* LPS (15 mg/kg, i.v.) and lethality was monitored for 72 h. In the five day pretreatment group only 9% of vehicle pretreated rats (N=22) survived the LPS challenge as compared to 87%

(N=23) and 80% (N=10) in the LPS and RD-LPS rats respectively ($P<0.05$). However, after four weeks resistance to LPS lethality abated and the number of survivors in the groups were comparable and not significantly different (20% vehicle control, N=10; 33% LPS tolerant, N=6; and 33% RD-LPS, N=6).

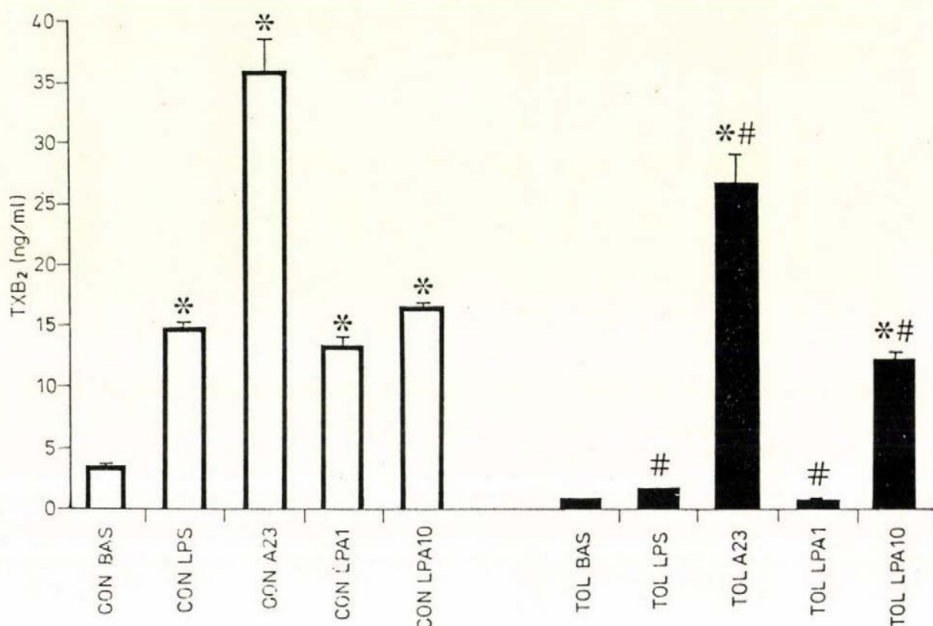


Fig. 4. Stimulated TXB₂ synthesis in macrophages 4 weeks after induction of tolerance with LPS. TXB₂ levels were measured in media from control (CON) and tolerant (TOL) macrophages at 3 h after LPS (50 µg/ml), calcium ionophore A 23187 (A23), (10 µM), lipid A (LPA) (1 µg/ml and 10 µg/ml). Data are expressed as mean $\pm$ S.E.M. N=3 separate experiments. * $P=0.05$ versus basal; # $P=0.05$ versus respective CON group

Discussion

These observations demonstrate that RD-LPS is equipotent with LPS in stimulating rat peritoneal macrophage TXB₂ production. The macrophage TXB₂ response to *in vitro* LPS and lipid A stimulation were markedly suppressed in cells harvested from rats subjected to the 5 day LPS or RD-LPS tolerance regimen. The calcium ionophore response was less attenuated presumably because calcium ionophore can bypass, in part, signal transduction changes induced by LPS tolerance [9, 10]. This suppressed macrophage response persisted for 4 weeks after the day 1 and day 2

sublethal doses of LPS or RD-LPS. Qualitatively the cells from the 4 week group were similar to the 4 day group. One exception was a tendency for recovery of the lipid A (10 $\mu\text{g/ml}$) induced TXB_2 response. The sustained reduction in macrophage AA metabolism could reflect long-term changes in differentiation of myeloid pools leading to monocytes or macrophages [11]. Indeed, studies by Hong et al. [12] have demonstrated persistence of LPS-induced suppression of AA metabolism for 2 months in peritoneal macrophages from rats administered *S. enteritidis* LPS.

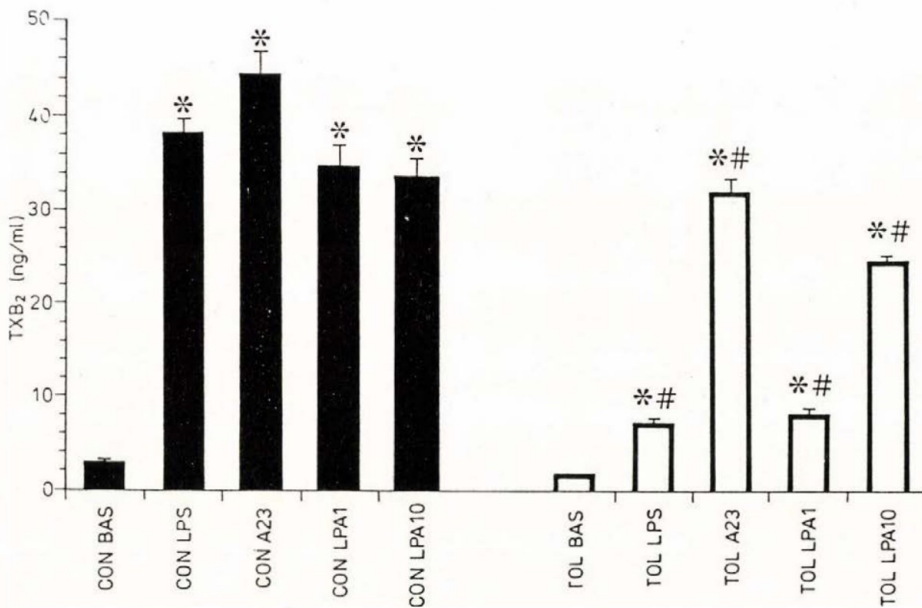


Fig. 5. Stimulated TXB_2 synthesis in macrophages at the fourth week after induction of tolerance with RD-LPS. TXB_2 levels were measured in media from control (CON) and tolerant (TOL) macrophages at 3 h after LPS (50 $\mu\text{g/ml}$), calcium ionophore A 23187 (A23), (10 μM), lipid A (LPA) (1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$). Data are expressed as mean $\pm$ S.E.M. $N=3$ separate experiments. * $P=0.05$ versus basal; # $P=0.05$ versus respective CON group

As with changes in macrophage mediator production, LPS and RD-LPS tolerance appeared to be equipotent in protecting rats from lethal LPS shock. Our previous studies have shown that LPS tolerance can be induced within 24 h after a single sublethal dose of endotoxin [9]. The LPS tolerance protection can persist for several days after sublethal endotoxin exposure as evidenced by the improved survival at 5 days in both LPS or RD-LPS-treated rats. Protection against LPS-induced lethality was not apparent after 4 weeks. These data thus demonstrate a disparity between peritoneal macrophage mediator production and persistence of in vivo lethality resistance. It is possible that the

difference between the *in vivo* and *in vitro* responses suggest that other populations of cells and/or mediators may more closely parallel resistance to lethality. Different tissue resident macrophage populations exhibit unique characteristics with regard to both mediator production and kinetics of response [13].

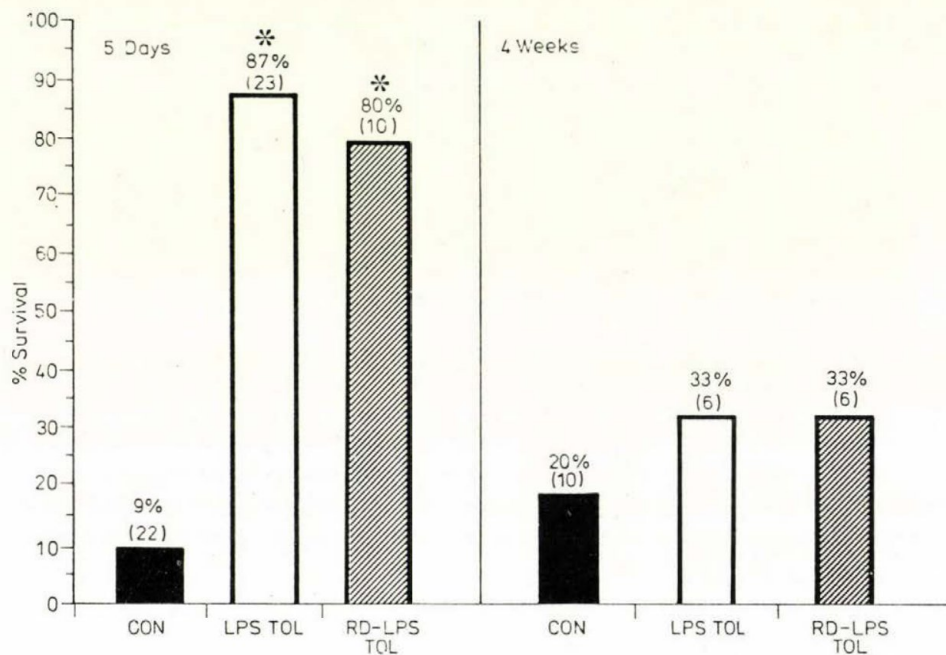


Fig. 6. Survival of rats when challenged with LPS at 5 days and 4 weeks after tolerance. Survival to LPS shock at 5 days and 4 weeks after induction of LPS tolerance with LPS or RD-LPS. * $P=0.05$ versus basal; # $P=0.05$ versus CON

Although peritoneal macrophages from LPS or RD-LPS tolerized rats were largely refractory to *in vitro* stimulation of TXB_2 with LPS or RD-LPS, this does not imply that production of other mediators is necessarily curtailed. Previous studies have shown that LPS tolerant macrophages exhibit selective alteration in inflammatory mediator production. In peritoneal MØ from LPS tolerant rats, LPS-stimulated thromboxane (TX) A_2 and 6-keto-prostaglandin (PG) $F_{1\alpha}$ [10, 14] are markedly suppressed, IL-6 is not greatly altered [12], whereas NO synthesis is augmented [15]. Increased NO and decreased $TNF\alpha$ responses to LPS are also observed in murine peritoneal MØ rendered tolerant to LPS [16]. Tolerance induction, both *in vivo* in mice [17], and *in vitro* in murine peritoneal MØ [18] suppresses $TNF\alpha$ but has little effect on

IL-1b. In human monocytes, LPS tolerance produces a more rapid down-regulation of TNF α than IL-1b, IL-6 and IL-8 [19]. Thus, there may be coexistence of discreet pathways of M ϕ desensitization for different mediators, accounting for differentially regulated mediator production in tolerance.

Cobalt 60-irradiated LPS produces degradation of the LPS molecule producing a mixture of diphosphoryl and monophosphoryl lipid A compounds and other LPS analogues [20, 21]. The reduced in vivo toxicity of RD-LPS while still retaining its immunoadjuvant properties makes this LPS preparation attractive as an agent to reduce tolerance. Indeed, RD-LPS has been shown to reduce tolerance not only to LPS but to mixed gram-positive, gram-negative and anaerobic bacterial infection [20–22]. The present study demonstrates RD-LPS is equipotent with native LPS in reducing macrophage alterations, and in lethality resistance during LPS tolerance induction. However, these observations suggest that during LPS or RD-LPS tolerance-induced suppression of LPS-stimulated AA in peritoneal macrophage metabolism persists longer than acquired lethality resistance.

Acknowledgements. This work was supported, in part, by NIH GM27673 and by NATO SA.5-2-05(CRG.9200754)1418/92JARC-501.

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SEROLOGICALLY VERIFIED HANTAVIRUS INFECTIONS IN HUNGARY

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(Received April 20, 1995)

(Accepted May 16, 1995)

Between 1987 and 1993 the etiological diagnosis of haemorrhagic fever with renal syndrome (HFRS) of 55 patients was confirmed using hantavirus-specific serology. The geographical distribution of cases indicated that at least two different territories of Hungary are endemic for hantaviruses. These possible natural foci are different from, but overlapping with the region endemic for tick-borne encephalitis virus. Patients sera were shown to react differently with reagents prepared from different hantaviruses, indicating that different types are present simultaneously in both natural foci.

Hantavirus infections have been recognized all over the world following the discovery of the disease in 1951 [1–4]. About 150 000 patients are hospitalized with haemorrhagic fever with renal syndrome (HFRS) each year [1]. Between 1978 and 1989 52 634 cases were clinically diagnosed in the former Soviet Union, 50 630 of them in the European, and 2 004 in the Asian region [2]. The annual morbidity rate in Bashkortostan (in the European part of Russia) was as high as 50 per 100 000 [5].

Epidemiological and clinical data indicated that rodents in Hungary are infected with the virus(es) [6, 7]. Epidemic form of hantavirus infections occurred in Hungary in the early 1950s, when small rural type epidemics evolved among soldiers stationed on military camps in late autumn periods [6, 7]. Although attempts to isolate the virus remained unsuccessful, retrospective laboratory investigations performed several years later in Bethesda, Maryland confirmed serologically the clinical and/or epidemiological diagnosis of HFRS observed in Hungary [8]. The number of recognized endemic and sporadic human illnesses were altogether 136 that appeared in 33 defined natural foci between 1952 and 1980 [19].

In the meantime distinct serotypes and antigenic variants of the hantaviruses have been identified [10–12], and isolated in the geographical surroundings of Hungary [3, 13, and personal communication of M. Labuda, Bratislava, Slovak Republic]. The

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recognized number of genetically distinct hantaviruses causing HFRS increased, too. In Slovenia and Small Yugoslavia (in the neighbourhood of Hungary) e.g. new hantaviruses have been recognized recently [3, 11–13]. More than 1000 HFRS cases have been registered since 1952 in the territory of the former Yugoslavia [13].

The occurrence of hantaviruses has been detected in rodents also on the American continent [14–16], nevertheless, human clinical diseases caused by hantavirus have been recognized only in 1993 [17, 18]. The different serotypes of previously known hantaviruses caused various clinical symptoms [4, 9, 14]. The new disease recognized in 1993 was the hantavirus associated pulmonary distress syndrome (HPS). This was characterized by acute bilateral pulmonary interstitial infiltration, and hypoxia followed by respiratory failure and frequent (60%) lethal outcome [17, 18].

The growing importance of the hantaviruses motivated the review of Hungarian hantavirus infections, which have been verified by different serological techniques during the last 5 years. The results presented indicate that different hantaviruses are persistently present in Hungary.

Materials and methods

Indirect immunofluorescent antibody technique (IFAT) was used at the beginning of the survey because of financial shortage and lack of laboratory with containment facilities required to handle these highly pathogenic viruses. Sera of 242 patients taken between January of 1987 and April 1993 were investigated by the IFA technique for hantavirus specific antibodies.

An antigen prepared from rat splenocytes infected with the H76-118 strain of Hantaan virus in Sofia, Bulgaria was used in the first year, nevertheless, later on a commercially available diagnostic kit have been purchased ("Hantavirus Antibody IF Assay" Progen Biotechnik GmbH, Heidelberg). This kit contains Puumala and Hantaan virus-infected Vero E6 cells as antigen fixed onto multiwell glass slides. The negative control was made from uninfected Vero E6 cells by the same procedure. Lyophilized positive and negative human control sera and FITC-conjugated goat anti-human IgG+IgM reagents were available in the kit. The tests were used according to the protocol suggested by the manufacturer. Because of economical reasons preselected sera were only tested (clinical diagnosis, i.e. suspected hantavirus infection, or renal disfunction and/or haemorrhagic symptoms with negative laboratory results for other pathogens e.g. leptospira, hepatitis A, B, C virus infections etc.). Sera were diluted to end point only in the case of special indication.

The high density silica particle (HDP) reagent coated with hantavirus antigens (HDP-A) was used in order to confirm our results by a different serological method (Hantadia/Korean Green Cross Corporation kit from the WHO Collaborating Center for Virus Reference and Research (HFRS) Institute for Viral Diseases, Korea University) was kindly provided by the manufacturer.

The Hantadia kit contained Hantaan virus antigen absorbed to coloured, high density silica particles (HDP-A), and the control-HDP was sensitized by normal rat brain components. Goat anti-Hantaan-virus serum served as positive control. Virus specific antibodies could be detected by direct particle agglutination reaction [20].

The freeze-dried positive and negative antigens required reconstitution for 30 min prior to use in order to form a suspension. Sera were diluted using Takátsy's microtiter system, and the positive and negative HDP(A) suspensions were pipetted into pairs of V-shaped wells similar to those of the original Takátsy's microtiter plates. The plates were thoroughly mixed (for 10 s) using a Titertec plate shaker. The agglutination patterns were read twice. First following one hour incubation at room temperature, then after an overnight standing at the room temperature according to the protocol given by the manufacturer.

This test can be applied either as a qualitative (screening) or as a quantitative procedure. In case of the qualitative application the sera had to be tested in 1:80 dilution with the negative and positive antigens simultaneously. In the case of quantitative application the end-point titre could also be determined [20, 21].

In order to obtain reliable results as much as possible sera taken from HFRS patients were tested, which had been collected between 1989 and 1993. Altogether 137 sera from 109 patients were reexamined by HDP test. The sera were inactivated at 56 °C for 30 min upon arrival at the laboratory and stored at minus 20 °C afterwards.

Results

Serum samples of 27 patients tested by the IFA technique proved to be positive for both IgG and IgM indicating an acute hantavirus infection. Altogether 225 preselected patients were investigated. The number of patients tested, and that of those with verified acute infections are shown in Fig. 1 as a function of the years of observation.

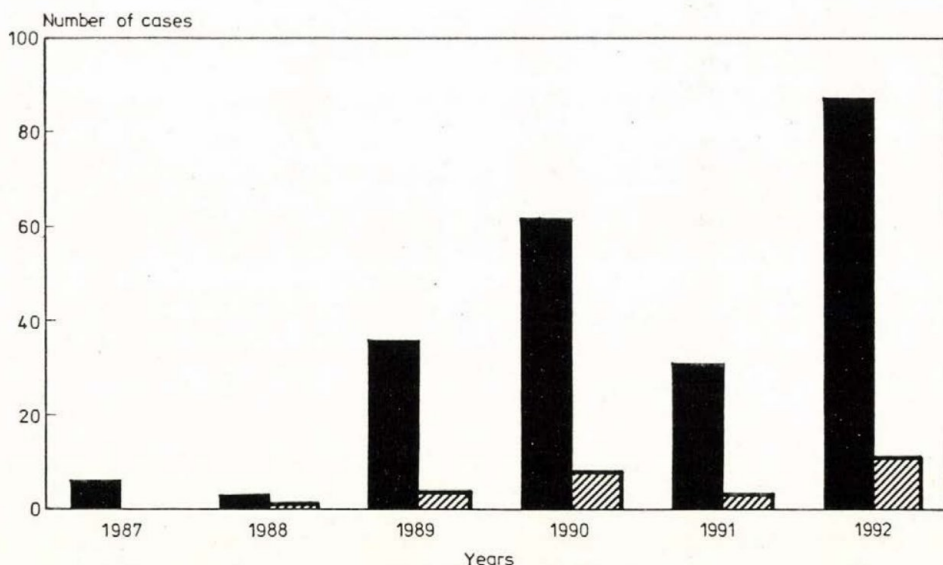


Fig. 1. Verification of hantavirus infections in Hungary using immunofluorescent antibody test. The number of patients tested between 1987 and 1992 (225) are shown by the black columns and the number of verified seropositive or seroconverted patients (27) are shown by the striated columns

The geographical distribution of acute cases is shown in Fig. 2. The numbers in Fig. 2 indicate the geographical distribution of 116 HFRS cases observed between 1953 and 1958. A few of them had been retrospectively verified by serological techniques [9]. Shadowing indicates the location of infections verified by both IFAT and HDPa serology. One or two cases were observed in 6 counties (dotted regions). Five or 6 patients were diagnosed in the capital, and in one county (striated regions). Only in one county were found more than 7 patients of positive serology verified by both techniques.

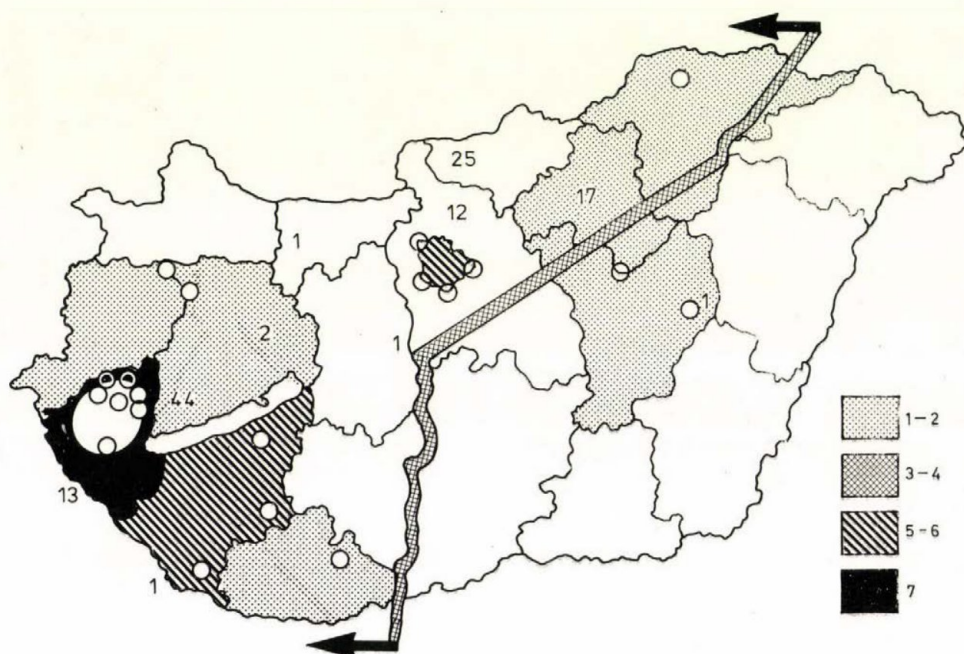


Fig. 2. Geographical distribution of the HFRS cases caused by verified hantavirus infection according to the residence of patients. Thin lines show the counties in the map of the Hungarian Republic. The number of cases verified by both IFAT and HDPa tests are shown by shadowing. The double-line terminated by arrows cutting the country into two halves indicates the border of the regions endemic (North-Western regions shown by arrows) and free (South-East from the line) of tick-borne encephalitis virus (modified on the basis of reference [22]). Circles indicate the locations of 20 infections, which could only be detected by HDPa. Numbers indicate the locations of 116 epidemic and sporadic cases observed between 1953 and 1959 taken from reference [7]. Only some of these cases had been retrospectively verified by serology [9]

Circles indicate residence of patients or the site of possible infections which could be verified only by the HDPa reagent. These data have been available only in the case of 20 infections. The majority of the diagnosed cases were observed at the contact

region of Slovenia, Hungary and Austria. Three counties situated along the Tisza river seem to represent a distinct ecological niche probably in close connection with a similar region of the Slovak Republic.

Table I

Results of serological investigation of serum samples taken from 109 patients between 1989 and 1993 in Hungary using IFAT and HDPA reagents

	1989		1990		1991		1992		1993		Total	
	+	-	+	-	+	-	+	-	+	-	+	-
HDPA												
IFA Positive	3	1	6	0	3	0	12	1	0	0	24	2
Inconclusive	0	0	5	0	7	4	11	23	3	3	26	30
Negative	0	0	0	1	0	0	3	17	2	4	5	22
Total	3	1	11	1	10	4	26	41	5	7	55	54

HDPA=High Density Particle Antigen

IFAT=Immunofluorescent Antibody Test

Four of the infections were imported, two of them from Roumania, one from the territory of the former Soviet Union and one from the former Yugoslavia.

The IFAT tests showed that 4 patients' sera reacted solely to Puumala (NEV=Nephropathia Epidemica Virus) antigens, 1 serum reacted to both Hantaan (HTN) and NEV antigens, all other positive sera reacted exclusively to HTN antigens.

HDPA tests showed good correlation with the IFAT, as far as the verification of IFAT seropositivity was concerned. Nevertheless, HDPA was found to be more sensitive. Altogether serum samples taken from 55 patients were found to be seropositive using the HDPA tests. Serum samples from 54 patients were found to be negative in the HDPA reaction. The comparative data of IFAT and HDPA tests are shown in Table I. One patient's sample who had been found IFAT-positive (shown in Fig. 1) was not available in 1993 and therefore it could not be retested with the HDPA reagent. Altogether two of the IFAT seropositive patients (one of them positive only with Puumala antigen) were found to be seronegative in the HDPA assay. Positive results of HDPA tests have only been accepted and presented, when the agglutination developed within one hour following the preparation of the incubation mixture.

The place of residence or that of the possible infection was registered in the case of 20 patients of the 31 whose infection was verified only by the positivity of HDPA reaction. These locations are also shown in Fig. 2 (circles). These infections occurred also within the regions found to be endemic for hantaviruses on the basis of previous epidemiological, and IFAT results [6, 7, 9].

HDPa results of overnight incubation indicated that 81 of the 137 sera retested with HDPa contained specific antibodies. Nevertheless, the grade of agglutination was found to be only one cross (+) in the case of 28 sera. Fifty-four gave two to four crosses (++ - ++++). The correlation between the 55 positive cases observed after one-hour incubation and the 54 cases which resulted in ++ to ++++ reaction following overnight incubation argue against the specificity of the very weak agglutination in the latter case. Although numerous clinical and laboratory findings in the majority of these 28 patients suggested the possibility of hantavirus infection, the weak positivity (+) was not accepted as a verification of the HFRS etiology in spite of the published data available concerning the specificity of the test [20, 21].

Discussion

The main purpose of this work was to verify the persistence of hantaviruses in Hungary, and to study the geographical distribution of human infections. The second serological technique, HDPa allowed the verification of 24 diagnoses obtained by different IFAT reagents previously.

Only two diagnoses could not be confirmed by HDPa. One of the two patients' sera with conflicting results showed positive reaction in IFAT exclusively with Puumala antigen and this fact could explain the negative finding observed with HDPa since it has been coated only with Hantaan virus antigen. In the case of the other patient, serum samples date back to 1988. Some storage failure and/or the antigen differences of different strains could be the possible reasons for the negative HDPa findings, since these sera reacted strongly and reproducibly with the Bulgarian rat splenocyte IF antigens earlier, and the positive reaction seemed to be specific.

The lack of cross-reaction with the commercial Puumala/Hantaanvirus IF antigens might indicate antigenic differences, too.

Antigenic differences of Hungarian Hantavirus strains have been suggested earlier on the basis of the results obtained previously by Takenaka et al. [8] who compared Hungarian patients' antibodies using IFAT and the plaque reduction technique in the laboratory of Dr. Gajdusek at the National Institutes of Health, Bethesda, Maryland. Comparison of their IFAT results with those of the plaque reduction neutralization tests with Hantaan virus revealed that convalescent sera following Hantaan virus infection did not react with IF antigens prepared from NEV or Prospect Hill viruses [8]. In contrast to this convalescent sera, which have been unable to neutralize Hantaan virus, reacted well with IF antigens prepared from all three (i.e. Hantaan, NEV and Prospect Hill) viruses used by them [8].

It has to be emphasized, that antibodies to Seoul virus cannot be detected with any of the antigens used in this study. The very weak agglutination (+) of HDPa particles following 24 h incubation with the sera of 28 patients suffering from HFRS-related disease might be also indicate that the causative agent could be a serologically different hantavirus serotype.

Our comparative studies showed that the HDPa kit was much more sensitive than IFAT. The geographical colocalization of the cases recognized only with HDPa

reagent confirm the specificity of the results (Fig. 2). There is, however, a contradiction in this respect in the paper published by Song et al. [21], who found very good correlation between positive results of HDPA and IFAT (98.4%, i.e. the specificity of the techniques was similar) although the antibody titres were shown to be significantly higher with HDPA than in IFAT, indicating lower sensitivity of the latter technique.

In this study only 24 of 55 HDPA positive patients were found to be positive with IFAT. More than the half of the Hungarian HFRS infections could not be diagnosed using immunofluorescence. Hantaanvirus-caused infections were shown to give very low or no cross-reaction with IF antigens prepared from other hantavirus types [8]. Samples of the 31 HDPA-positive patients, however, were unreactive or gave inconclusive (Table I) results with an IF antigen composed of both Puumala and Hantaan viruses. Taking into account that the two methods have been shown to possess similar specificity in the case of Hantaan virus [21], it can be concluded that the 31 infections were caused by a hantavirus different from Puumala and Hantaan viruses, but cross-reacting with Hantaan virus antigen-coated HDPA particles.

The geographical distribution of the cases revealed that at least two ecological niches can be identified in Hungary. The distribution of hantavirus-infected regions are overlapping, but not identical to those endemic for the tick-borne encephalitis virus causing the most important arboviral infection in the country [22]. Seropositivity to West Nile virus and other arboviruses has been also observed in different regions, but no clinical illnesses were associated with the seropositivity [23].

The regions South-East from the thick line drawn through the country in Fig. 2 are free of tick-borne encephalitis. In contrast to TBE several hantavirus infections were serologically verified within this region. Numbers in the map indicate the geographical location of 116 epidemic and endemic HFRS cases observed between 1953 and 1958 [6, 7]. These data indicate that the relative frequency of human infections decreased in the Northern counties, but slightly increased in the Southern counties of Hungary. The presented serological results suggest that at least three types of Hantaviruses are enzootic in Hungary.

In 1994 two lethal illnesses occurred resembling pulmonary distress syndrome in Hungary (Gy. Princz personal communication). Only one of them was found to be seropositive by IFAT (data not included in this study). In order to collect further information on the different hantaviruses of this regions virus isolations, examination of rodents, and systematic investigation of the natural foci will be necessary.

Acknowledgements. We should like to thank Professor Ho-Wang Lee M.D. for personal help in providing the HDPA kit and methodological and practical advices, and to Dr. B. Komarintsev, Academy of Military Medicine, Sofia, for the IFAT reagents prepared from Hantaanvirus-infected rats. We gratefully appreciate the encouragement and help of Drs György Berencsi and Ilona Mezey. The enthusiastic technical assistance of Mrs M. Kaposi is also acknowledged. We wish to thank Professor István Dömök for the critical reading of the manuscript. The expenses of the research were partly covered by the Hungarian Scientific Research Fund (OTKA), Grant No. 785/1991-1994 of the and by the Grant No. 784 of the Research Fund of the Hungarian Health Insurance.

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ROLE OF NITROGEN FIXING BACTERIA ON THE PHYLLOSPHERE OF WHEAT SEEDLINGS

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(Received May 9, 1995)

(Accepted May 19, 1995)

Two diazotrophic bacteria *Azotobacter chroococcum* (REN₂) and *Corynebacterium* sp. (Pot N₂) isolated from rice and potato phyllosphere, respectively, were tested on wheat seedlings in vitro, for their efficiencies to increase dry weight and total nitrogen content of the plant, to establish their utility as potential biofertilizers. An average increase in dry weight nearly by 35–50% and total nitrogen content by 56–52% was obtained which was near to that available by nitrogenous chemical treatment. A comparatively better result was obtained by REN₂ than Pot N₂.

Although the role of all the phyllospheric microorganisms on higher plants cannot be assessed from literary data, it is at least clear, that the phyllosphere nitrogen fixers play a role in the nitrogen economy of the host plant in some cases [1–5]. In tropical countries with low nutrient availability in the soil, the leaf surface inhabiting bacteria may greatly enrich the nutritional status of the plants by fixing nitrogen of the atmosphere [6]. The fixed nitrogen may be utilized by the plants either by direct absorption of the excreted nitrogenous products of the bacteria through leaves or from soil enriched by the products of such organisms [7].

The utility of the application of the phyllosphere nitrogen fixers for agronomic purposes has not been adequately explored. The total amount of fixed nitrogen is insignificant in most of the cases due to the scanty presence of the nitrogen fixer on the leaf surfaces in nature. If the frequency of such organism on the leaf surface can be increased through foliar spray of their cultures at appropriate growth stages of the crop plants, the total nitrogen content of the host plant will be considerably increased. Previously Vasantharajan and Bhat [8] observed that the inoculation of mulberry leaf and root (in water culture) with *Azotobacter* sp. increase the growth, total nitrogen content and dry weight of the host plant. Similar reports have been published by El-Hoseiny et al. [9] in barley and maize plants.

In the present study, to establish their utility as potential biofertilizers, the isolated nitrogen fixers were tested in vitro for their efficiencies to increase the dry weight and total nitrogen content of wheat seedlings. This was done with wheat seedlings grown in mineral nutrient solution and sprayed with the selected diazotrophs

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and nitrogenous chemicals over the leaves of wheat seedlings either separately or mixed with the nitrogen fixing cultures.

Materials and methods

Bacterial strains and culture medium. Two previously isolated phyllospheric bacteria [10] *Azotobacter chroococcum* (REN₂) and *Corynebacterium* sp. (Pot N₂) were selected for this study and were grown and maintained in Burk's nitrogen free broth and agar medium (in g/litre K₂HPO₄ 0.64, KH₂PO₄ 0.16, MgSO₄·7H₂O 0.20, NaCl 0.20, FeSO₄·7H₂O 0.003, CaSO₄·2H₂O 0.05, Na₂MoO₄·2H₂O 0.001, sucrose 10.0, agar 15.0)

Preparation of inocula. The strains were grown in 100 ml conical flask/each containing 25 ml Burk's broth and incubating at 30 °C for 48 h on a rotary shaker (speed 30 rpm). These cultures were used to inoculate 100 ml of fresh, sterilized Burk's broth distributed in 250 ml conical flasks. The inoculated flasks were incubated at 30 °C 72 h on a rotary shaker, then the inocula were centrifuged at 10 000 g for 20 min. The cells were resuspended in 50 ml of fresh Burk's broth without sugar. The suspensions were then used for spraying.

Preparation of the experimental set. Fresh wheat seeds (CV Sonalika) were surface sterilized with 0.1% mercuric chloride for 5 min followed by washing repeatedly with sterile distilled water before they were placed on wet filter paper within sterile Petri dishes for germination. After 48 h, uncontaminated germinated seeds were placed within the cotton plugs of sterile culture tubes fitted with a side arm. The tubes had been previously filled with sterile Hogland mineral nutrient solution (Table I). Roots of the seedlings were always kept immersed in the nutrient solution. Two seeds were placed within the cotton plug of each tube. Different sets, each consisting of 10 tubes, were prepared for testing the effects of spraying the two organisms and different nitrogenous chemicals on the leaves of the seedlings. While the seedlings continued growing, fresh sterilized Hogland solution was added to each tube through the side arm. The experimental sets were kept under proper light condition and temperature for one month.

Treatment schedule. Different treatments (T₁–T₁₁) were made by spraying the following suspensions on the leaves of wheat seedlings in separate experimental sets: T₁ = water (control); T₂ = only medium (sterile Burk's broth); T₃ = supernatant after bacterial growth; T₄ = cell suspension of the strain REN₂; T₅ = cell suspension of the strain Pot N₂; T₆ = (NH₄)₂SO₄ solution (0.5%); T₇ = urea solution (0.25%); T₈ = T₄ + T₆ (1:1 v/v); T₉ = T₄ + T₇ (1:1 v/v); T₁₀ = T₅ + T₆ (1:1 v/v); T₁₁ = T₅ + T₇ (1:1 v/v).

One set was kept as control by spraying the leaves with water (T₁) only. The spraying was done with the help of a small, sterilized glass sprayer, fitted with a rubber tube and blower. Spraying was done after every seven days until the seedlings attained the age of one month.

Recording of data and statistical analysis. The average lengths of roots and shoots of the seedlings, in all the sets, were recorded after one month. The length of the longest root in each seedling was taken into consideration. The number of rootlets on the root were considered and the number of leaf in each plant were counted. The plants in each set were then dried in an oven and the dry weight was measured accurately. Finally, the total nitrogen contents of the dried plants, treated with different spray solutions, were measured by the conventional microkjeldahl method for nitrogen estimation. All the readings for different treatments were compared with those of the control and the percentage of increase and decrease in the weight of dry matter and total nitrogen content of the plants over the control was calculated in each case. All the data were statistically tested for significance Wilk's test [11].

Results

After one month, root length, shoot length, number of rootlets and number of leaves in each seedling were recorded (Table II). Maximum growth of root, shoot and leaf, as compared with those of control seedlings, were noticed in seedlings, treated with only urea solution. Next, in order of beneficial effect on the growth of wheat seedlings was ammonium sulphate. Spraying of experimental sets resulted in a marked increase in the visible growth over that of the control plants. There was practically no effect of the uninoculated medium and supernatant after bacterial growth on any of the characteristic of the plant. However, in this respect the strain REN₂ was much more effective than strain Pot N₂. Any of the two organisms mixed with urea solution induced slightly better growth than the single spray culture of the corresponding organism. The same trend was noticed in case of cultures mixed with (NH₄)₂SO₄ solution. The additive effects, however, were not very significant. Any organism mixed with urea, or (NH₄)₂SO₄ solution, when sprayed on the leaves of the seedlings, enhanced the growth of the wheat seedlings in decreasing order. However, the effect of any of the mixed spray solutions in most cases was little lower than that of the single solution of the corresponding chemical fertilizer. The degree of enhancement of growth of wheat seedlings, sprayed with nitrogen fixer REN₂ was very close to those sprayed with (NH₄)₂SO₄ solution.

Table I

Composition of modified Hoagland mineral nutrient solution

Compound	Concentration of stock solution, g/litre	Volume of stock solution per litre of final solution, ml
KNO ₃	101.10	6.0
Ca(NO ₃) ₂ ·4H ₂ O	236.16	4.0
NH ₄ H ₂ PO ₄	115.08	2.0
MgSO ₄ ·7H ₂ O	246.49	1.0
KCl	3.728	1.0
H ₃ BO ₃	1.546	1.0
MnSO ₄ ·7H ₂ O	0.338	1.0
ZnSO ₄ ·7H ₂ O	0.575	1.0
CuSO ₄ ·5H ₂ O	0.125	1.0
H ₂ MoO ₄	0.081	1.0
Fe-EDTA	6.922	1.0

Enhancement in the weight of dry matter and the total nitrogen content of wheat seedlings, due to the treatment with different single and mixed spray solutions, were expressed as percentage of increase over the control (Table III). The organisms REN₂

and Pot N₂ in single spray solutions increased the dry weight of wheat seedlings by nearly 50% and 35%, respectively, as compared to the control. The percentage of total nitrogen increase in wheat seedlings over the control, due to the effect of REN₂ and Pot N₂ were almost 62% and 56%, respectively. Regarding the impact on the increase of both dry weight and total nitrogen content, the organisms REN₂ closely resemble (NH₄)₂SO₄. So far as these two parameters of growth were concerned, the foliar spray of urea solution was most effective of all spray solutions. Although culture Pot N₂ increased the dry weight and total nitrogen content of wheat seedling significantly over the control, the effect was much lower than that of REN₂ and the nitrogenous chemicals. In most of the cases the selected nitrogen fixers, mixed with any of the chemical fertilizers, when sprayed on the leaves of wheat seedlings, resulted a little more weight of dry matter and total nitrogen content of host seedlings than being sprayed in single culture solution. The effect of mixed spray solution of a nitrogen fixing culture and a nitrogenous chemical on the total dry weight and total nitrogen content of wheat seedlings were less than those of the corresponding nitrogenous chemical solution.

Table II

Effect of the isolated phyllospheric diazotrophs and different chemical fertilizers on the growth of wheat seedlings in liquid culture

Treatment**	Average length, cm		Average number of	
	root	shoot	rootlets/root	leaf/plant
T ₁	10.6	14.8	4	3
T ₂	10.6	14.6	4	3
T ₃	10.6	14.9	4	3
T ₄	16.2*	22.5*	7*	6*
T ₅	14.1*	18.5*	6*	5*
T ₆	17.3*	24.0*	7*	6*
T ₇	15.4*	26.0*	8*	7*
T ₈	17.0*	23.5*	7*	6*
T ₉	18.0*	25.0*	9*	6*
T ₁₀	16.8*	21.5*	6*	5*
T ₁₁	17.0*	24.5*	7*	6*
SE =	±0.07	±0.21	±0.37	±0.33
CD at 5% =	0.20	0.61	1.06	0.95

* Significant at 5% level

** T₁=only water (control); T₂=only medium(Burk's broth); T₃=supernatant after bacterial growth; T₄=REN₂ cell suspension; T₅=Pot N₂ cell suspension; T₆=(NH₄)₂SO₄ solution; T₇=urea solution; T₈=REN₂ + (NH₄)₂SO₄ solution; T₉=REN₂ + urea solution; T₁₀=Pot N₂ + (NH₄)₂SO₄ solution; T₁₁=Pot N₂ + urea solution

Table III

Effect of the isolated phyllospheric nitrogen fixers and different nitrogenous chemical fertilizers on the dry weight and total nitrogen content of wheat seedlings in liquid culture

Treatment*	Average dry weight, in mg/plant	Percent increase (+) or decrease (–) of dry weight over control	Average nitrogen content, mg/plant	Percent increase (+) or decrease (–)
T ₁	16.61	–	0.34	–
T ₂	16.77	0.96 (+)	0.33	2.94 (–)
T ₃	16.62	0.06 (–)	0.34	–
T ₄	24.55*	47.80 (+)	0.55*	61.76 (+)
T ₅	22.22*	33.77 (+)	0.53*	55.88 (+)
T ₆	25.50*	53.52 (+)	0.57*	57.64 (+)
T ₇	26.79*	61.28 (+)	0.61*	79.41 (+)
T ₈	24.50*	47.50 (+)	0.57*	55.88 (+)
T ₉	26.15*	57.43 (+)	0.59*	73.52 (+)
T ₁₀	23.25*	30.97 (+)	0.52*	52.90 (+)
T ₁₁	24.25*	45.99(+)	0.52*	52.90 (+)
SE = ± 0.17		SE = ± 0.008		
CD at 5% = 0.24		CD at 5% = 0.024		

* Significant at 5% level

** Treatments T₁–T₁₁ : see notes of Table II

Discussion

The potentiality of the phyllospheric nitrogen fixers as a source of nitrogenous nutrients for growing crops in field could not be assessed properly. Ruinen [1, 12] was the first to suggest that a well established and mixed phyllosphere population could make a substantial contribution to the nitrogen requirement of field vegetation. She also suggested that the vegetation benefits not only by the foliar uptake of the fixed nitrogen but also by the absorption through roots of the leaf wash which follows a heavy dew or rain. Bessems work [13], however gave the first clear evidence with plants that the phyllosphere may prove to be one of interesting zones of plant-microbe interaction.

The present study, although in vitro experiments differ from field conditions, revealed the possibility of success of using these diazotrophs in large scale as ready biological source of nitrogen. This supports the observation of our previous work [5], and work of Gupta et al. [3].

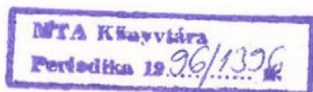
Another aspect of this study was that the nitrogenous chemicals like urea, $(\text{NH}_4)_2\text{SO}_4$, which are mostly used in soil to increase soil fertility can also be alternatively used on the leaves of growing crop plants as a foliar spray and hence contribute nitrogen directly to the plants.

Of the two nitrogen fixing organisms, REN_2 (*A. chroococcum*) has been proved to be much more effective in increasing the growth of the wheat plants in water culture. This supports the observations of several workers [9, 10, 14] on different plants, sprayed with *A. chroococcum*. The potentiality of the other nitrogen fixing strain Pot N_2 (*Corynebacterium* sp.) in increasing the growth of wheat plants was quite significant as compared with the unsprayed control plants. However, no worker has yet been able to establish this organism as an appropriate biofertilizer. So the successful use of different strains of this organism in the field, as a substitute of chemical fertilizer would be an important step towards the improvement of agriculture in the country with low soil nitrogen.

The effect of the selected stain Pot N_2 in increasing the dry weight and total nitrogen content of wheat plants was, however, much lower than REN_2 . This is explained by the fact than REN_2 fixed nitrogen at a higher rate than Pot N_2 [15]. The degree of enhancement of growth, dry weight and total nitrogen content of wheat plant by the foliar spray of REN_2 (*A. chroococcum*) resembled closely that obtained by the foliar spray of $(\text{NH}_4)_2\text{SO}_4$. Sengupta et al. [16] made a similar observation. Cumulative effect of the mixed spray solution of a nitrogen fixing organism and a nitrogenous chemical, was always lower than the effects of the single solutions of the corresponding chemical fertilizer. It may be assumed that in mixed spray solutions, some amount of the nitrogenous chemicals were utilized or degraded by the nitrogen-fixing bacteria. Ruinen [6] also showed that the cultural condition for active nitrogen fixation quickly deteriorated by the accumulation of metabolic products both from the leaf and the micro-vegetation. The cumulative effect of the mixed spray solution of nitrogen fixing organism and nitrogenous chemical, was always higher than that of the corresponding organism. This observation supports the report of Meshram and Shende [14] who described that the total nitrogen uptake (kg/h) by maize plants in the field, inoculated with *A. chroococcum* combined with moderate application of N-fertilizer, was influenced significantly and resulted in a higher total nitrogen content and better yield. But El-Hoseiny et al. [9] reported a contradictory observation revealing that the pattern of stimulation of growth, dry weight and total nitrogen content of barley and mize plants, inoculated on the phyllosphere by *A. chroococcum*, decrease when the plants were fortified with urea.

From this in vitro experiment it may be expected that the two isolated diazotrophs would be able to survive and contribute significant amount of nitrogen on the phyllosphere of growing wheat plants under field condition. Although the beneficial effects of phyllospheric diazotrophs were little less than those of synthetic fertilizers, the former are worth using to avoid the possible hazards of using chemical fertilizers employed in large quantity.

Acknowledgement. The authors are grateful to the UGC for financial assistance.



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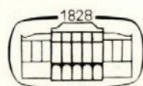
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ACTA MICROBIOLOGICA ET IMMUNOLOGICA HUNGARICA

Volume 42

NUMBER 1

B Cell Specific Natural IgM Autoantibodies: Multifunctional Regulators of the Humoral Immune Response (A Review)	
<i>Uher, F., Kiss, K., Rácz, J., Mihalik, R., Puskás, É., Alonso, M. E.</i>	3
Growth of Untreated and Radiation-Damaged <i>Listeria</i> as Affected by Environmental Factors	
<i>Farkas, J., Andrásy, É., Mészáros, L., Bánáti, D.</i>	19
Production and Some Characteristics of β -Glucosidase in <i>Diaporthe (Phomopsis) Helianthi</i>	
<i>Peričin, D., Jarak, M.</i>	29
Epifluorescent Microscopy of Earthworms' Intestinal Bacteria	
<i>Krištúfek, V., Pižl, V., Ravasz, K.</i>	39
Bacteria Adherent to the Hindgut of Terrestrial Isopods	
<i>Drobne, D.</i>	45
Hybrid Process for Ethanol Production from Rice Straw	
<i>Chadha, B. S., Kanwar, S. S., Saini, H. S., Garcha, H. S.</i>	53
Effect of Some Environmental Factors on <i>Rhizobium</i> and <i>Bradyrhizobium</i> Strains	
<i>Bayoumi, H. E. A. F., Bíró, B., Balázs, S., Kecskés, M.</i>	61
Simultaneous Saccharification and Fermentation of Rice Straw into Ethanol	
<i>Chadha, B. S., Kanwar, S. S., Garcha, H. S.</i>	71
A Simple and Rapid Semiquantitative Method for Measuring Cellulase Activity in Agar Media	
<i>Jakucs, E., Várallyay, É.</i>	77
HLA Associated Cytotoxic Antibody Production in Dialyzed Chronic Uraemic Patients	
<i>Kovanecz, I., Petri, I., Kaiser, G.</i>	81
Diversity in the Antigenic Structure of Different Hexon Types among the Members of Adenovirus Subgenus D	
<i>Ádám, É., Nász, I., Lengyel, A.</i>	85
Annual Meeting of the Hungarian Society for Microbiology	93

NUMBER 2

Evolutionary Aspects, and Taxonomic Definition of Viruses together with Mobile Extrachromosomal Elements of Relative Autonomy into a Special Highest Rank Taxon (A Review)	
Berencsi, Gy., Bánrévi, A., Takács, M., Lengyel, A., Nász, I.	141
Nutrient Optimization for Production of Broad Spectrum Antibiotic by <i>Streptomyces antibioticus</i> Sr _{15.4}	
Haque, S. F., Sen, S. K., Pal, S. C.	155
In Vitro Suppression of Anti-TSH Receptor Antibody by Autologous Anti-Idiotypic Antibody in Patients with Graves' Disease	
Balázs, Cs., Molnár, I.	163
Effects of Some Heavy Metals on Growth, Pigment and Antibiotic Production by <i>Streptomyces galbus</i>	
Raytapadar, S., Datta, R., Paul, A. K.	171
Altered Humoral Immunity against Cytomegalovirus and Epstein-Barr Virus without Detectable Virus Antigens and Virus-DNA in the Glomeruli of Patients with IgA Nephropathy in Remission Phase	
Nagy, J., Haikin, H., Sarov, B., Háber, Á., Kun, L., Sarov, I.	179
Longitudinal Immunological Follow-up of HIV Infected Haemophiliacs in Hungary	
Ujhelyi, E., Králl, G., Zimonyi, I., Bánhegyi, D., Hollán, S. R., Horváth, A., Fuchs, D., Wachter, H., Berkessy, S., Füst, G.	189
Remarks on the Appearance of "Violette" on Cantal Cheese (A Note)	
Chaballier, C., Ratomahenina, R., Galzy, P., Dieu, B.	199
Immunomodulating Effect of Acridine Tautomers on Eukaryotic Cells	
Petri, I. B., Berek, I., Galy, A.-M., Barbe, J., Berek, L., Molnár, J.	203
The Effect of Vasopressin on the Phosphoinositides of <i>Tetrahymena pyriformis</i> . Relationship with Glycogen Content	
László, V., Csaba, G.	209
A Model for Testing Drug Susceptibility of <i>Pseudomonas aeruginosa</i> and <i>Staphylococcus aureus</i> Grown in Biofilms on Medical Devices	
Kétyi, I.	215
Biofilms Produced by <i>Pseudomonas aeruginosa</i> and by <i>Staphylococcus aureus</i> on Model Medical Devices	
Kétyi, I.	221
Dehydroepiandrosterone Modulates the Spontaneous and IL-6 Stimulated Fibrinogen Production of Human Hepatoma Cells	
Fehér, K. G., Rákász, É., Bíró, J., Falus, A.	229
Detection of Hepatitis C Virus (HCV) by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (A Note)	
Falus, A., Hajnal, A.	235

NUMBER 3

Immunological Responses to Phospholipase-A of <i>Salmonella typhi</i> Punj, V., Hittu Matta, Kalra, M. S.	237
An Adenovirus-Herpes Simplex Virus Glycoprotein B Recombinant (Ad-HSV.gB) Protects Mice against a Vaccinia HSV.gB and HSV Challenge Endresz, V., Berencsi, K., Gönczöl, E.	247
Comparison of Petrifilm™ Method to Conventional Methods for Enumerating Aerobic Bacteria, Coliforms, <i>Escherichia coli</i> Yeasts and Molds in Foods Jordano, R., Lopez, C., Rodriguez, V., Cordoba, G., Medina, L. M., Barrios, O.	255
Interaction of Immunomodulant Substances in Mouse Experiments with Special Regard to Drug Sensitivity and Bacterial Translocation Andlerik, P., Szeri, I., Antmann, K., Bános, Zs.	261
The Number of Glucocorticoid Receptors in Peripheral Human Lymphocytes is Elevated by a Zinc Containing Trace Element Preparation Falus, A., Béres, J. Jr.	271
Interaction between Tricyclic Psychopharmacons and Some Antibiotics Molnár, J., Bathó, N., Csík, V., Chevalier, J., Cremieux, A.	277
Resistance to Beta-Lactam Antibiotics and Beta-Lactamase Production of <i>Bacteroides</i> , <i>Porphyromonas</i> and <i>Prevotella</i> Strains Nagy, E., Szóke, I., Gacs, M., Csiszár, K.	287
Effects of Pentoxifyllin and Pentaglobin® on TNF and IL-6 Production in Septic Patients Mándi, Y., Farkas, Gy., Ocsóvszky, I.	301
The Entire <i>rne</i> Gene: The Remaining Questions Miczák, A.	309
Complement C4, Factor B and C3 Polymorphism in North India Kramer, J., Srivastava, L. M., Ferenczy, E., Füst, G.	315
Study on the Glycoprotein Nature of Chicken IFNS by Chemical Cleavage and Inhibition of Glycosylation Taródi, B., Pusztai, R., Solymosi, T., Béládi, I.	321

NUMBER 4

Lactic Acid Production from Molasses by <i>Sporolactobacillus cellulosolvens</i> Kanwar, S. S., Tewari, H. K., Chadha, B. S., Punj, V., Sharma, V. K.	331
Antibodies to Spotted Fever Group Rickettsiae and <i>Coxiella burnetii</i> among Domestic Animals in Southern Croatia Punda-Polić, V., Poljak, S., Bubić, A., Bradarić, N., Klišmanić-Nuber, Z.	339
Kinetic Characterization of Thyroid Peroxidase Enzyme and its Inhibition by Autoantibodies Kaczur, V., Vereb, Gy., Molnár, I., Krajczár, G., Balázs, Cs.	345
Seroepidemiologic Study of Anti-HAV IgG in Health-Care Workers Kassas, A. L., Mihály, I.	351
Comparison of Selected Species of <i>Bipolaris</i> , <i>Drechslera</i> and <i>Exserohilum</i> by Random Amplification of Polymorphic DNA Bakonyi, J., Pomázi, A., Fischl, G., Hornok, L.	355
Oxygen and Carbon Dioxide Requirements of <i>Helicobacter pylori</i> (A Note) Narikawa, S., Imai, N., Yamamoto, M., Suzuki, T., Yanagawa, A., Mizushima Y.	367

Microbial Counts and Characteristics of Streptovorticillium Strains Isolated from Soils in the Jordan Valley Mahmoud, J. I., Abussaud	373
Antibiotic Resistance of <i>Acinetobacter calcoaceticus</i> Strains Isolated from Patients Treated in Intensive Care Units Kiss, L., Leskó, T., Széll, M.	381
Is Seminal Fluid a Suitable Specimen for Detecting Chlamydial Infection in Men? Corradi, Gy., Konkoly Thege, M., Pánovics, J., Molnár, Gy., Bodó, Á., Frang, D.	389
Interaction Between <i>Bordetella pertussis</i> Vaccine and Chlorpromazine in Mouse Experiments Antmann, K., Anderlik, P., Ohár, A., Szeri, I., Bános, Zs.	395
A Higher Production of Platelet Activating Factor in Ex Vivo Heterologously Secondary Dengue-2 Virus Infections Yang, Kuender, D., Lee, Chuen-Shin, Shaio, Men-Fang	403
Radiodetoxified Endotoxin-Induced Tolerance. Effect on Endotoxin Lethality and Macrophage Arachidonic Acid Metabolism Bertók, L., Takáts, A., Hong Chen, Cook, J. A.	409
Serologically Verified Hantavirus Infections in Hungary Faludi, G., Ferenczi, E.	419
Role of Nitrogen Fixing Bacteria on the Phyllosphere of Wheat Seedlings Pati, B. R., Sengupta, S., Chandra, A. K.	427

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